Chapter 6

Proteomic Analysis of Frozen Tissue Samples Using Laser Capture Microdissection

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Abstract

The discovery of effective cancer biomarkers is essential for the development of both advanced molecular diagnostics and new therapies/medications. Finding and exploiting useful clinical biomarkers for cancer patients is fundamentally linked to improving outcomes. Towards these aims, the heterogeneous nature of tumors represents a significant problem. Thus, methods establishing an effective functional linkage between laser capture microdissection (LCM) and mass spectrometry (MS) provides for an enhanced molecular profiling of homogenous, specifically targeted cell populations from solid tumors. Utilizing frozen tissue avoids molecular degradation and bias that can be induced by other preservation techniques. Since clinical samples are often of a small quantity, tissue losses must be minimized. Therefore, all steps are carried out in the same single tube. Proteins are identified through peptide sequencing and subsequent matching against a specific proteomic database. Using such an approach enhances clinical biomarker discovery in the following ways. First, LCM allows for the complexity of a solid tumor to be reduced. Second, MS provides for the profiling of proteins, which are the ultimate bio-effectors. Third, by selecting for tumor proper or microenvironment-specific cells from clinical samples, the heterogeneity of individual solid tumors is directly addressed. Finally, since proteins are the targets of most pharmaceuticals, the enriched protein data streams can then be further analyzed for potential biomarkers, drug targets, pathway elucidation, as well as an enhanced understanding of the various pathologic processes under study. Within this context, the following method illustrates in detail a synergy between LCM and MS for an enhanced molecular profiling of solid tumors and clinical biomarker discovery.

Key words Biomarker, Cancer, Laser capture microdissection (LCM), Liquid chromatography-mass spectrometry (LC-MS), Solid tumor heterogeneity, Frozen tissue

1 Introduction

At present, very few cancers can be cured and clinical outcomes for the majority of tumor types remain disappointing. What are sorely needed are novel molecular diagnostics and improved medications. However, new 'omics technologies (genomics, transcriptomics,

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proteomics, metabolomics) are transforming our understanding of disease and are helping to provide new therapeutic options (1). In this regard, future discovery of effective biomarkers through advanced molecular profiling is promising.

Recently, a new molecular diagnostic for multiple myeloma was developed and is now available commercially (2–4). Critical in the success of this assay development was the use and analysis of purified human subject cell populations, via flow cytometry. This approach permitted the heterogeneity and complexity of a hematologic/liquid malignancy to be reduced, thus enhancing effective scientific study, as well as assay development for clinical use. For solid tumors, laser capture microdissection (LCM) can be viewed as an analog to flow cytometry (5).

Solid tumors possess a heterogeneous cellular architecture. Significant heterogeneity exists among tumors of the same organ system as well as within individual tumor samples (6). Recently, intratumor heterogeneity has been effectively illustrated as a significant clinical issue (7). Thus, there is little surprise why the categorical results of large clinical trials are far from optimal when applied to individual patients. Additionally, new treatments based on devising a therapy regimen from a genetic test that is derived from an outpatient tumor biopsy appear to be a bit too simplistic (8).

Fundamentally, the salient elements of solid tumors include cancer cells proper along with cellular and structural stromal elements. The histology may be quite complex; for instance, an epithelial tumor may contain regions of carcinoma in situ, well to poorly differentiated carcinoma, inflammation, and neovascularity. The tumor microenvironment is composed of both normal and modified stromal cells that serve to nurture the malignant process. The tumor stroma is now recognized as an important area in cancer therapy and many new therapeutic strategies target aspects of this functional region (9).

Solid tumor heterogeneity is reflective of the diversity present at the molecular level and has profound biologic and therapeutic implications (10). For instance, breast cancer is actually many different diseases with the only common characteristic being the organ of origin. Hence, the ability to directly and effectively profile solid tumors at the proteome level is essential, since proteins are the final mediators of pathologic processes, and proteomics in particular can begin to characterize fundamental molecular events such as alternative protein splicing and post translational modifications. Additionally, although cell culture and model organism studies are quite important, they lack a true microenvironment, and invariably the cells utilize some different biochemical systems and clinical translation can be limited (11, 12). Therefore, methods to decompose solid tissue to better enable biological understanding and biomarker discovery are needed (13–15).

LCM and MS (16) are powerful independent analytical technologies. Both have been commonly used for molecular profiling of formalin-fixed paraffin-embedded tissue sections (17). Additionally, we have shown that the LCM-MS platform can be effectively used for proteomic profiling of thin fresh-frozen tissue sections obtained from a solid tumor in conjunction with a simple methanol-aided (18) solubilization and digestion process (19). Furthermore, we advocate that fresh frozen tissue can be particularly useful in some circumstances to avoid potential bias (20). In this chapter, we further illustrate this method for profiling the proteomes of a solid tumor using LCM coupled to biological MS for clinically relevant biomarker discovery.

2 Materials

2.1 LCM

1. TISSUE-Tek O.C.T. cryostat mounting medium (Sakura Finetek Inc., Torrance, CA).
2. Frozen tissue staining: Mayer's hematoxylin solution, eosin Y solution (alcohol-based), and Scott's tap water substitute bluing solution (magnesium sulfate buffered with sodium bicarbonate).
3. Frozen tissue dehydration: 100 % ethanol (ethyl alcohol, absolute, 200 proof for molecular biology). 70 % (v/v) and 95 % (v/v) ethanol baths were prepared using Milli-Q filter with purified water. Xylene is used in the final post dehydration step.
4. CapSure® Macro LCM Caps (Life Technologies, Applied Biosystems, Carlsbad, CA).
5. PixCell IIe, Veritas, or ArcturisXT (Life Technologies, Applied Biosystems, Carlsbad, CA).
6. Leica Cryostat CM 1850 UV, Leica Microsystems, Wetzlar, Germany.
7. Pre-cleaned glass microscope slides, 25 × 75 mm.
8. Membrane slide options include:
   (a) Pen-membrane glass slide.
   (b) Pen-membrane frame slide; both options available from Life Technologies, Applied Biosystems, Carlsbad, CA.

2.2 Protein Extraction and Digestion

1. Sequencing grade trypsin (Promega, Madison, WI, USA).
2. ZipTips packed with C_{18} reversed-phase resin (Millipore, Billerica, MA, USA).

2.3 Reverse-Phase LC-MS

1. LC-MS buffers: Buffer A consists of 0.1 % FA in purified H_2O. Buffer B consists of 0.1 % FA in HPLC grade acetonitrile (ACN).
2. MS sample rehydration with 0.1 % trifluoroacetic acid (TFA).

2.4 Computational Support for CID Spectra Analysis

1. Single computer workstation or a cluster computer that follows a Beowulf design model (see Note 1).
2. Software for protein database search and match to experimental mass spectrometry data (see Note 2).
3. Non-redundant human proteome database.
4. Software for reverse database creation for the assessment of a false-positive rate.
5. Software to analyze experimental data for biologic classification and implications (see Note 3).

3 Methods

Handling the tumor sample rapidly and effectively during the tissue acquisition step is critically important in order to obtain reliable downstream molecular results. Tissue degradation and possibly frank necrosis can begin once a solid tumor is ligated from its blood supply. Thus, a few simple but deliberate steps are recommended to minimize ischemic effects. As quickly as possible, the tissue should be snap frozen in liquid nitrogen and then placed in a freezer at –80 °C. Embedding the tumor tissue in cryostat-mounting medium (TISSUE-Tek O.C.T.) can then be performed.

Tissue sections, usually with a slice thickness range of 8–12 μm, are then serially cut from the frozen tissue block using a cryostat. As a convenient measurement, the majority of cells will have a diameter either larger than or within the range of the thickness of the section. Therefore, the recommended slice thickness will aid in the homogenization/lysis procedure. Clearly, a key aspect of this method is maximizing the effective liberation of proteins from captured cells.

MS is a critical component in any bottom-up proteomic analysis. Key sample handling factors such as effective lysis and digestion are requisites for effective large-scale protein identification. Optimal buffering conditions are required for successful digestion of small-size LCM specimens. Keeping proteins solubilized and denatured throughout the digestion process is essential; thus avoiding unnecessary manipulations and/or use of reagents that might interfere with LC-MS analysis is certainly advocated.

We deliberately chose to simplify and improve the analysis of LCM captured cells, and thus avoid the deficiencies associated with traditional approaches, which typically employ detergents or chaotropes. Hence, a simple two-step methanol-assisted solubilization and digestion protocol was developed. In the first step, 20 % buffered methanol is used to facilitate denaturation and solubilization of cytosolic proteins. In the second step, the digestion is carried out in a 60 % methanol buffer, targeting more hydrophobic proteins that are insoluble in 20 % buffered methanol. We have found

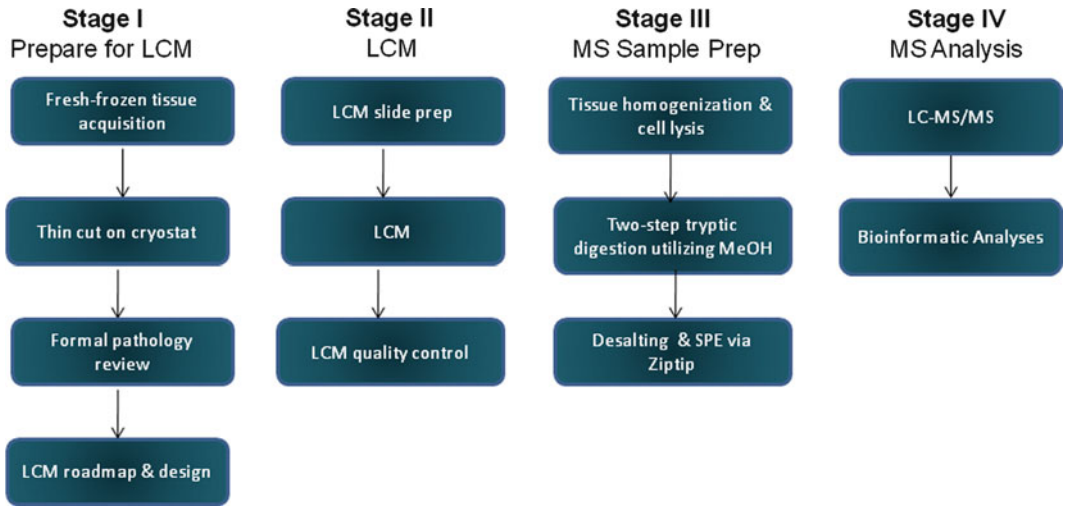

Fig. 1 Laser capture microdissection-mass spectrometry experimental design

this two-step approach results in enhanced proteome coverage. A comprehensive schematic of this experimental workflow, broken down into four stages, is illustrated in Fig. 1. Note: the use of organic solvents for micro and nanoscale proteomic sample prep methods has been very successfully pioneered by other groups (21).

3.1 Initial Pathologic Analysis (Prior to LCM)

A formal hematoxylin and eosin (H&E) staining procedure with cover slip should be performed using every tenth slide. Prior to LCM analysis, these slides should be reviewed with a pathologist to properly evaluate the histology, plan LCM sessions, and guard against potential bias in the *z*-dimension of the tumor tissue plane.

3.2 LCM Staining

The fresh frozen tissue slide must be fully defrosted before beginning the staining protocol. Placing the slide in the palm of your glove works well. As soon as condensate forms on the entire slide the protocol below may commence. To ensure good visualization and tissue capture, suggested times are provided for both membrane and glass slides (see Note 4).

Step	Solution	Comment	Time (membrane slide)	Time (glass slide)
1	70 % ethanol	Fix tissue section to slide	15 s	30 s
2	d.d. water	Remove OCT, rehydrate tissue	30 s	30 s
3	Hematoxylin	Stain nuclei	45 s	30 s
4	d.d. water	Remove excess hematoxylin	15 s	30 s
5	Bluing solution	Change hematoxylin hue	15 s	30 s

(continued)

Step	Solution	Comment	Time (membrane slide)	Time (glass slide)
6	70 % ethanol	Start dehydration	15 s	30 s
7	Eosin	Stain cytoplasm (1–2 quick dips)	1–2 s	2 s
8	95 % ethanol	Dehydration	30 s	1 min
9	95 % ethanol	Dehydration	30 s	1 min
10	100 % ethanol	Dehydration	30 s	2 min
11	100 % ethanol	Dehydration	30 s	2 min
12	Xylene	Ethanol removal	3 min	3 min

3.3 LCM Procedure

LCM analysis may begin on the slide(s) once they are air-dried. Typically, laser-based systems allow for dissections approaching 100 % purity. Staining with H&E allows microscopic visualization during microdissection and does not diminish protein recovery. Generally, we have found that depending on the type of tissue under study, approximately 5,000–50,000 cells are required to produce MS results with acceptable numbers of protein identifications, as well as protein class diversity (see Note 5). Figure 2 illustrates a stepwise approach for successful LCM tissue extraction, which is typical of either a PixCell IIe or Veritas system. LCM tissue extraction involves:

1. Establishing a histology area of interest (Fig. 2a).
2. Manual filling of the pattern to enable removal of cells (Fig. 2b).
3. LCM extraction of the cells from the selected region (Fig. 2c).

3.4 LCM Sample Prep Protocol

The sample preparation protocol for protein extraction and digestion from LCM samples captured on polymer cap is presented below.

3.4.1 Phase I: LCM Membrane-Based Tissue/Cell Extraction and Lysis

1. Prepare a hypotonic lysis buffer (ammonium bicarbonate to methanol: v/v = 80/20, pH ~ 8.0). For convenience, we recommend a 1 mL stock solution prepared by mixing 800 µL of 12.5 mM ammonium bicarbonate (final concentration) with 200 µL of 100 % MeOH and 2 µL of 0.5 mM TCEP (1 mM final concentration).
2. Carefully remove the LCM polymer membrane by peeling it off the CAP and then place it in a new siliconized tube (conical bottom).
3. Add 50 µL of hypotonic lysis buffer.
4. Incubate on dry ice for 30 min.

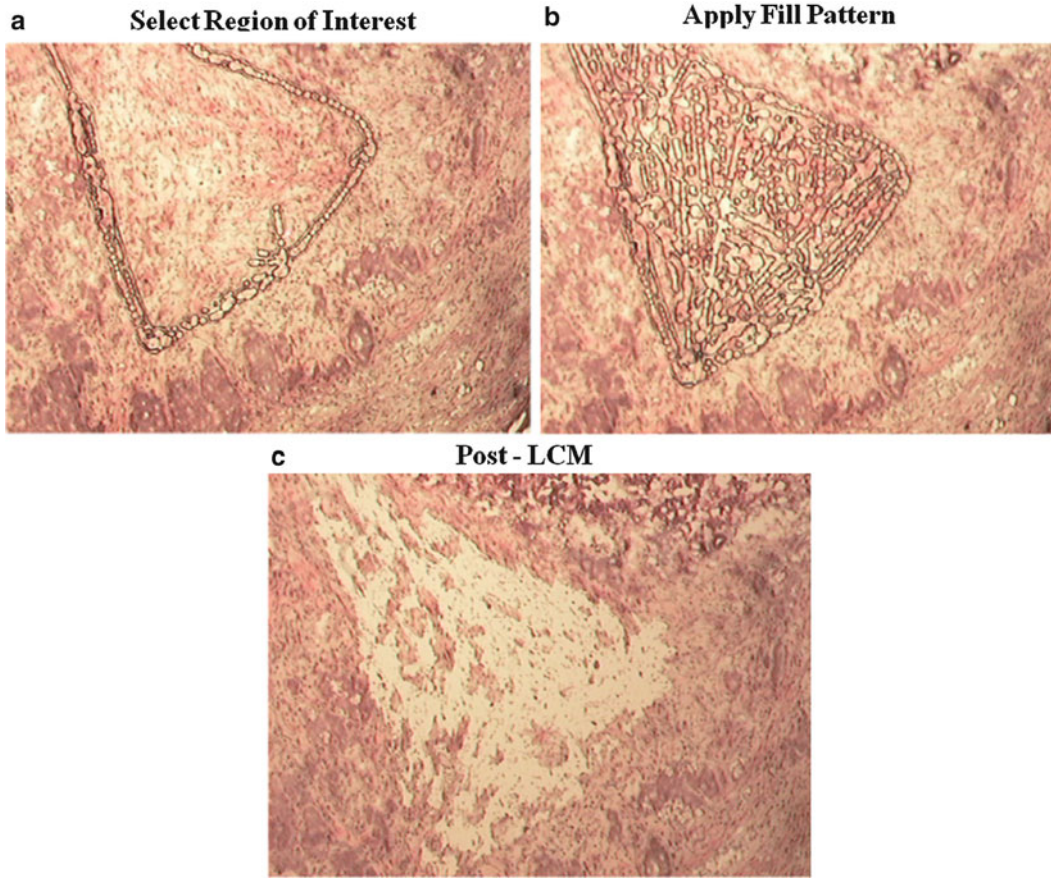

Fig. 2 Laser capture microdissection workflow

5. Thaw the sample in ice-cold water for 10 min.
6. Incubate the sample in a water bath for 2 h at 70 °C.
7. Cool the sample on ice for 20 min.
8. Adjust the buffer from 12.5 to 50 mM by adding 1.65 μL of 1 M ammonium bicarbonate.

3.4.2 Phase II: Initial Trypsin Digestion (Ratio of Trypsin:Protein = 1:50)

The background to the trypsin dilution protocol is based on single-cell protein content estimates in the range of 0.75 pg to 0.5 ng (see Note 6).

Prepare the dehydrated and frozen trypsin, e.g., Promega Trypsin Gold, 20 μg vial.

Mix with 20 μL of 50 mM ammonium bicarbonate, yielding a concentration of 1 μg/μL.

The availability of some tissue samples is quite limited in quantity. Therefore, this section attempts to accommodate these circumstances as well as situations with more abundant tumor tissue.

Recommended amount of trypsin as a function of sample cell count.

Cell count	Protein estimate (μg)	Trypsin:protein	Trypsin for sample (μg)
50	0.025	1:50	0.0005
500	0.25	1:50	0.005
5,000	2.5	1:50	0.05
15,000	7.5	1:50	0.15
50,000	25	1:50	0.5

1. Rehydrate trypsin by adding 20 μL of 50 mM ammonium bicarbonate, pH ~ 8.0.
2. Dilute trypsin in accordance with sample cell count per the trypsin dilution protocol.
3. Add the appropriate volume of trypsin solution and mix for 10 min.
4. Briefly vortex the sample.
5. Place the sample in the water bath sonicator for 5 min.
6. Transfer the sample tube to a small centrifuge and spin for ~15 s.
7. Incubate the sample digest for 6 h at 37 °C with good table motion.

3.4.3 Phase III: The Second Trypsin Digestion (Ratio of Trypsin:Protein = 1:20)

1. Make a 60 % methanol buffer (50 mM ammonium bicarbonate + 100 % methanol (v/v 40/60)). Add the appropriate volume of trypsin solution and mix for 10 min.
2. Briefly (~5 s) vortex the sample.
3. Place the sample in the water bath sonicator for 5 min.
4. Transfer the sample tube to a small centrifuge and spin for ~15 s.
5. Incubate the sample for 6 h at 37 °C with good table motion.
6. Lyophilize all samples to dryness.

3.4.4 Phase IV: Desalting Using ZipTip Columns (Solid Phase Extraction)

1. Rehydrate peptides in 20 μL 0.1 % TFA by sonication in a water bath for 2 min.
2. Prepare 10 μL aliquots of elution buffer consisting of 60 % ACN/0.1 % TFA (v/v) for each sample before beginning (to avoid contamination). Avoid drawing air through the tip during the procedure (from equilibration to elution). If you find that you make bubbles in the tip, try pulling the buffers in more slowly.
3. Set the Pipetman to 10 μL and attach the ZipTip.
4. Activate the ZipTip column by pipetting 20 μL of 60 % ACN and then discarding it to waste. Repeat this process three times.
5. Equilibrate the ZipTip column by pipetting 20 μL of 0.1 % TFA and then discarding it to waste. Repeat this process three

times. These steps act as a gradient for the mini-column, which activates the resin and conditions it to bind peptides.

6. Load the peptides by pipetting the sample up and down (discarding it back into its tube). Repeat this process ten times.
7. Wash the ZipTip column using the 0.1 % (v/v) TFA. Pipette up the solution and then discard it to waste. Repeat this process ten times.
8. Elute the sample by pipetting the ZipTip up and down in the elution buffer back into its tube in the 4 μL 60 % ACN/0.1 % TFA (already aliquoted). Repeat this process ten times. The organic phase elutes the peptides off the resin into the buffer. The sample is desalted as well as concentrated.
9. Lyophilize to dryness and dissolve the peptides in 10 μL of 0.1 % (v/v) TFA prior to LC-MS/MS analysis.

3.5 Guidelines for LC-MS Analysis of LCM Samples

Although there are a wide variety of mass spectrometer systems and liquid chromatography platforms, a linear ion trap mass spectrometer coupled with a reverse-phase liquid chromatography separation system is widely used in the proteomics community, and will be illustrated in this section. For our LCM-based proteomic studies, we utilize a reversed-phase column coupled directly on-line with a linear ion trap mass spectrometer (LTQ ThermoElectron, San Jose, CA). Certainly, newer instruments such as the Orbitrap have higher mass accuracy (1–2 ppm), resolving power, and dynamic range. This improves both the depth and integrity of the discovery process. This protocol is fully compatible with such instruments.

1. In our configuration, the solvent system is delivered using the HP 1100 pump (Agilent Technologies, Palo Alto, CA).
2. A nano-electrospray ionization source is employed applying a voltage of 1.7 kV, and a capillary temperature of 160 °C.
3. The LTQ is operated in a data-dependent mode in which the seven most abundant peptide molecular ions detected by each MS survey scan are dynamically selected. They are then passed for MS/MS (fragmentation) using collision-induced dissociation (CID) facilitated by a normalized collision energy of 35 %.
4. Dynamic exclusion is employed to avoid redundant acquisition of precursor ions previously selected for fragmentation.
5. Reversed-phase liquid chromatography separations are performed using a 75 μm i.d. × 10 cm long fused silica capillary column (Polymicro Technologies, Inc., Phoenix, AZ) with a flame-pulled tip (~5–7 μm orifice).
6. The column is slurry packed in-house with 5 μm, 300 Å pore size C-18 stationary phase (Vydac, Hercules, CA) using a

slurry-packing pump (model 1666, Alltech Associates, Deerfield, IL) (see Note 7).

7. Note: the total MS run time for each sample is 180 min.
   (a) After injecting 5 μL of sample, the column is washed for 30 min with 98 % mobile phase A (0.1 % FA in d.d. water).
   (b) Peptides are then eluted using a linear step gradient from 2 to 40 % mobile phase B (0.1 % FA in ACN) over 90 min.
   (c) Then, an elution gradient of 60–98 % for mobile phase B over 10 min at a constant flow rate of 0.25 μL/min is performed.
   (d) Next, the column is washed for 20 min with 98 % mobile phase B.
   (e) Finally, the column is re-equilibrated with 2 % mobile phase B for 30 min prior to subsequent loading of the next sample.

3.6 Data Processing Guidelines

As previously stated, the searching and matching of experimentally obtained spectra against a non-redundant protein database is computationally intensive, but highly parallelizable and therefore amendable to "divide and conquer" strategies employing cluster computers.

1. For our LTQ-derived data, the precursor ion tolerance is set to 1.5 Da, and the fragment ion tolerance to 0.5 Da. These two values effectively serve as binning parameters during data acquisition concerning parent and daughter (fragment) ions.
2. We require candidate peptides to possess tryptic terminus at both ends, and generally will allow for a maximum of two missed tryptic cleavages.
3. The following SEQUEST thresholds are routinely used to filter experimental peptides:
   (a) Delta-correlation score (dCn) ≥ 0.08.
   (b) Charge state cross correlation scores as follows:
   ≥2.1 for $[M+H]^{+}$ peptides.
   ≥2.3 for $[M+H]^{2+}$ peptides.
   ≥3.5 for $[M+H]^{3+}$ peptides.
4. The final list of protein identifications is created using a parsimony principle, reporting a minimal number of protein identifications from a pool of uniquely identified peptides.
5. Resultant raw data are routinely subjected to a false-positive rate assessment via decoy (reverse) database analysis (22).
6. In the final step the data are analyzed for biologic implications by Ingenuity Pathway Analysis and the Database for Annotation, Visualization, and Integrated Discovery (DAVID).

4 Notes

1. The processing of CID spectra is computationally intensive but highly parallelizable. Therefore, a cluster computer solution generally offers substantial time-savings. This approach follows a linear function that is dependent on the number of computational elements in the cluster configuration. Multicore computers may likely offer similar speed advantages as software tools become more adept in parallel task operations and interactions.
2. Commercial products include MASCOT (Matrix Science, http://www.matrixscience.com) and SEQUEST (Thermo Scientific, http://www.thermo.com). Open source solutions include (a) the X! Tandem database search engine (http://www.thegpm.org/tandem), (b) the Trans-Proteomic Pipeline (TPP, http://tools.proteomecenter.org/wiki/index.php?title=Software:TPP), and (c) the open mass spectrometry search algorithm (OMSSA) (pubchem.ncbi.nlm.nih.gov/omssa/).
3. Commercial products include Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com). Public domain tools include the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov). Innovative bioinformatic approaches towards the analysis of LCM-MS data are essential for progress. Along these lines, a quite interesting computational approach has been put forth by Karger and Sgroi (23).
4. For each step in the staining protocol, a different solution bath is recommended. Through experience this procedure has been found to make a significant difference to subsequent analyses. Additionally, when glass slides are used for LCM, the time for dehydration (steps 8–12) may need to be increased up to 1 min (occasionally up to 3 min) for each ethanol bath. The increased time may improve the pickup of captured cells from the glass slide onto the LCM Cap. Finally, enhanced dehydration is usually not required for membrane slides.
5. We have found that tissues with a compact cellular density provide greater protein yields and thus usually require a smaller quantity of cells. However, when encountering a new tumor tissue type, a few preliminary experiments are recommended to determine the general estimate of protein yield.
6. In addition to the provided reference, these estimates are also cited in the following text books: Molecular Biology of the Cell, third edition, by Alberts et al. and Molecular Cell Biology, fourth edition, by Lodish et al.
7. This protocol does not depend on custom columns. Adequate high-quality commercial columns are available (e.g., New

Objective, Inc.; http://www.newobjective.com). Additionally, some groups (Karger et al.) have developed custom high-pressure columns, which potentially result in an enhanced separation. Such innovations are vital for the improved analysis of minute samples, as is characteristic with LCM (24, 25).

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