Chapter 2

Laser Microdissection and Saturation Labeling DIGE Method for the Analysis of Human Arteries

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Abstract

Laser microdissection (LMD) is a novel methodology for noncontact isolation of tissue regions or cells for subsequent molecular analysis. Although it is an upcoming field, its combination with proteomics for differential analysis remains not very well explored, since amount of protein obtained after LMD is scarce. We have combined LMD arterial layer isolation with saturation labeling DIGE, successfully achieving differential analysis of healthy and pathological intima and media layers. Identification of differential spots could be performed in whole tissue extract as reference proteome, since studied regions are subproteomes of the aforementioned.

Key words Laser microdissection, Coronary artery, Radial artery, Aorta artery, Atherosclerosis, Proteomics, Saturation labeling DIGE

1 Introduction

Events provoked by coronary atherosclerosis constitute the main cause of death in developed countries. Although our knowledge of atherosclerosis pathogenesis is constantly growing (1), the underlying molecular mechanisms remain uncertain. Tissue analysis of coronary artery, where the pathology arises, provides interesting information of the molecular mechanisms responsible for atherosclerosis development (2). Since whole tissue analysis may be very complex, pre-fractionation of the tissue in its layers allows better localization of occurring molecular events. In this way, laser microdissection is the best approach to perform such fractionation, since it constitutes a fast, reproducible, and noncontact isolation methodology (3). The combination of LMD with proteomics has the difficulty of a relatively poor protein yield after LMD, since regions excised are thin and of limited surface area. The sensitivity of mass spectrometers has exponentially increased in the last years (4, 5), allowing the analysis of scarce samples as LMD extracts. In addition,

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combination of LMD and protein microarrays has been set up successfully and offers interesting possibilities in the field of differential proteomic analysis (6). Moreover, differential analysis based on 2-DE methodology has overcome this limitation by means of saturation labeling of cysteine residues with fluorochromes (7), which very significantly augments fluorescence of the proteins and provides high-resolution spot maps with very scarce total protein amounts.

Previous studies on coronary artery layers have focused on describing the proteome of the three composing layers of the artery by means of LC-MS/MS (8). We have developed a methodology to isolate such layers by laser microdissection and pressure catapulting (LMPC) and compare them with healthy arteries by means of 2D-DIGE. To date, we have provided the first 2-DE spot maps of arterial intima and media layers (9), and the one 2D-DIGE analysis of intima layer of atherosclerotic coronary, where the pathology mostly develops, revealing altered proteins implicated in the migrative capacity of vascular smooth muscle cells (VSMCs), extracellular matrix (ECM) composition, coagulation, apoptosis, heat shock response, and intraplaque hemorrhage deposition (10).

2 Materials

Aqueous and partially aqueous solutions used during the staining procedure for LMD have to be supplemented with 0.01 % protease inhibitor cocktail (Sigma-Aldrich) and precooled at 4 °C, unless otherwise specified. DTT (BioRad) and Pharmalytes pH 3–10 or pH 4–7 (GE Healthcare) should be added in the moment of using sample and rehydration buffers.

2.1 Histology

1. Optimal Cutting Temperature compound (OCT, Sakura Finetek).
2. Antibodies: Anti-smooth muscle actin antibody, Clone 1A4 (Dako), anti-CD68 antibody, Clone PG-M1 (Dako), REAL Antibody Diluent (Dako), secondary antibody: peroxidase-conjugated EnVision + Dual Link (ready-to-use solution, Dako).
3. Mayer's hematoxylin solution (Sigma-Aldrich), Eosin Y alcoholic solution (Sigma-Aldrich), 70 %, 95 %, and 100 % ethanol, xylene.
4. DPX mounting medium for microscopy (Merck).
5. Formaldehyde solution for molecular biology, 36.5 % (Sigma-Aldrich).
6. Oil Red stock solution: 1 g Oil Red O (Sigma-Aldrich), add 2-propanol (Sigma-Aldrich) up to 100 ml.
7. Glycerol gelatin (Sigma-Aldrich).

8. Hydrogen peroxide 3 %, from hydrogen peroxide 33 %.
9. Wash buffer 1×: From wash buffer 10×, Dako.
10. Blocking solution: BSA 10 % in wash buffer 1×.
11. Liquid DAB+ substrate-chromogen system (Dako).

2.2 Tissue Processing and LMD Isolation

1. Saline solution: Sodium chloride 0.9 % (Braun).
2. Optimal Cutting Temperature compound (OCT, Sakura Finetek).
3. Polyethylene naphthalate (PEN) membrane slides (PALM Microlaser, Carl Zeiss).
4. 70 % and 100 % ethanol (Merck).
5. Certistain Cresyl violet (Merck).
6. Microbeam system (PALM Microlaser, Carl Zeiss).
7. 500 µl opaque adhesive cap tube (PALM Microlaser, Carl Zeiss).
8. Lysis buffer: 7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris, 1 % DTT (*see* **Note 1**).
9. Protein Desalting Spin Column (Pierce).
10. Acetone HPLC-grade (Scharlau).

2.3 Saturation Labeling DIGE Buffers

1. Labeling buffer: 7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris, pH 8.0.
2. pH Fix 7.5–9.5 indicator strips (Macherey-Nagel).
3. 50 mM sodium hydroxide (NaOH) solution, 50 mM hydrochloric acid (HCl) solution.
4. 24 cm IPG strips pH 4–7 (GE Healthcare).
5. Rehydration buffer: 7 M urea, 2 M thiourea, 4 % CHAPS, 1 % Pharmalytes pH 4–7 and 13 mM DTT.
6. 2 mM TCEP solution (Sigma-Aldrich).
7. CyDye DIGE Fluor Labelling Kit for Scarce Samples (GE Healthcare).
8. 2× sample buffer: 7 M urea, 2 M thiourea, 4 % CHAPS, 2 % Pharmalytes pH 3–10 and 130 mM DTT.
9. Protean IEF Cell (BioRad), Ettan Dalt Six (GE Healthcare), 9400 Typhoon Scanner (GE Healthcare).
10. Equilibration buffer: 6 M urea, 50 mM Tris, 30 % glycerol, 2% SDS, pH 8.8.
11. DeCyder 2D Differential Analysis Software v. 7.0 (GE Healthcare).
12. 20 mM TCEP solution.
13. Fixation solution: 30 % ethanol, 5 % acetic acid in bidistilled water.

14. Silver Staining Kit, Protein (GE Healthcare).
15. DP protein digestion station (Bruker-Daltonics).
16. Ammonium bicarbonate (Sigma-Aldrich) 50 mM and 20 mM.
17. 50 % methanol (Sigma-Aldrich), 15 % 2-propanol, 60 % and 30 % acetonitrile, 0.1 % trifluoroacetic acid.
18. 20 ng/μl porcine trypsin (Promega), α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich).
19. 384 Opti-TOF 123×81 mm MALDI plate (AB Sciex), 4800 Plus MALDI TOF/TOF Analyzer (AB Sciex).

3 Methods

3.1 Arterial Sample Collection and Histology (Fig. 1)

1. Wash the tissue with saline at least three times or until blood contamination is eliminated. Dry the specimen with 100 % cellulose paper and place it on an adequate cast for embedding. Add OCT and freeze it with liquid nitrogen. Store it at −80 °C until use (*see* **Note 2**).
2. Cut 5 μm sections, consecutive to the ones to be used for LMD, with a cryostat for histological analysis.
3. Hematoxylin and eosin staining: Fix in cold acetone (−20 °C) for 5 min, remove OCT with tap water, stain in hematoxylin solution for 10 min, wash in running tap water, dip two to three times in eosin solution, dehydrate in ethanol 70 %, 95 %, 100 % solutions, clear in xylol, and mount in DPX.
4. Oil Red O staining: Fixate tissue with 4 % formaldehyde in PBS. Prepare working solution by filtrating 12 ml of stock solution and adding 8 ml of distilled water. Stain slides with working solution for 20 min (*see* **Note 3**). Rinse with distilled water. Stain with hematoxylin for 1 min. Warm glycerol gelatin at 50 °C to melt it and mount the slides (*see* **Note 4**).
5. Actin immunohistochemistry: Fix in cold acetone (−20 °C) for 5 min, remove OCT with tap water, mark selected area with ImmunoPen, incubate with blocking solution for 1 h, followed by smooth muscle actin antibody 1:500 in antibody diluent for 30 min, wash with wash buffer, eliminate peroxidases activity with hydrogen peroxide 3 % for 5 min, wash with wash buffer, incubate with secondary antibody for 30 min, wash with wash buffer, incubate with DAB (5–10 min), wash with water (*see* **Note 5**), stain in hematoxylin solution (30 s to 1 min), wash in running tap water, dehydrate in ethanol 70 %, 95 %, 100 % solutions, clear in xylol, and mount in DPX.
6. CD68 immunohistochemistry: Follow actin staining protocol with anti-CD68 as primary antibody.

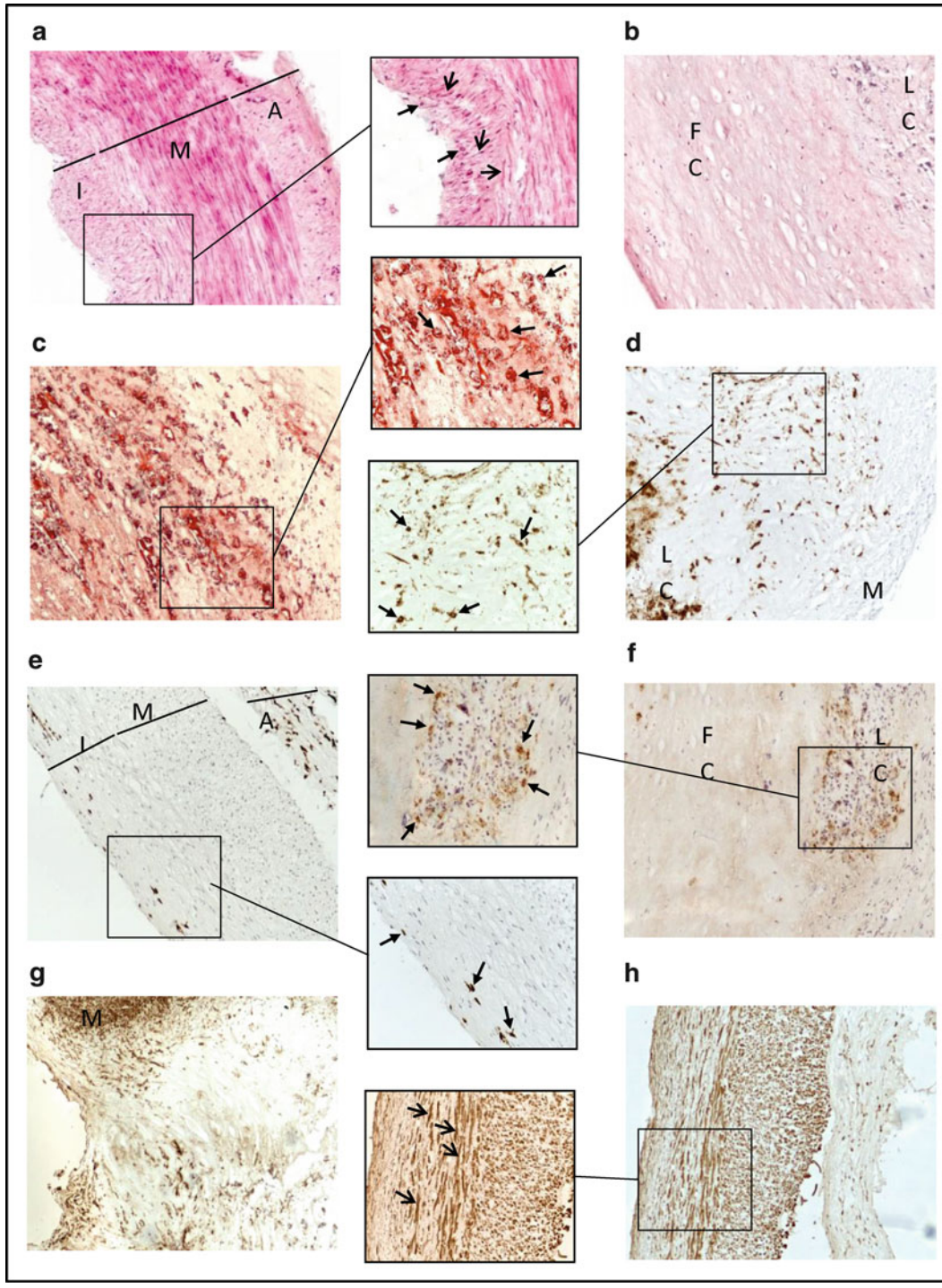

Fig. 1 Histology of atherosclerotic and preatherosclerotic arteries. H&E and Oil Red staining, together with CD68 and actin IHC, were performed with every artery studied in order to identify the artery architecture and to characterize their atherosclerotic lesion degree. (**a**) Preatherosclerotic radial biopsy, H&E. (**b**) Atherosclerotic coronary autopsy, H&E. (**c**) Atherosclerotic coronary autopsy, Oil Red. (**d**) Atherosclerotic coronary biopsy, CD68. (**e**) Preatherosclerotic coronary autopsy, CD68. (**f**) Atherosclerotic coronary autopsy, CD68. (**g**) Atherosclerotic coronary biopsy, actin. (**h**) Preatherosclerotic coronary autopsy, actin. In some images, a subregion has been augmented (200× magnification). *I* intima, *M* media, *A* adventitia, *LC* lipid core, *FB* fibrous cap. *Open-end arrow*: VSMCs. *Closed-end arrow*: macrophages/foam cells. From (10) with permission

3.2 Processing for LMD Isolation

1. Cut 8–10 μm sections with a cryostat and place them on 3 PEN membrane slides (*see* **Note 6**).
2. Fixate the slides with −20 °C precooled 70 % ethanol. Let dry completely on ice and rinse with bidistilled water at room temperature to remove OCT. Dehydrate with 70 % and 100 % ethanol (*see* **Note 7**).
3. Stain for 1 min with Cresyl violet in 100 % ethanol (*see* **Note 8**). Wash staining and dehydrate with 70 % and 100 % ethanol. Place slides on ice until LMD is performed.

3.3 Laser Microdissection (Fig. 2)

1. Check arterial architecture with the 5× objective and select the regions of interest with the 10× objective for a more accurate delimitation. Select the exact number of regions needed to isolate 8 mm^2 of tissue (*see* **Note 9**).
2. Check laser energy power and focus for cutting and catapulting by isolating 2–3 delimited elements before starting automatic

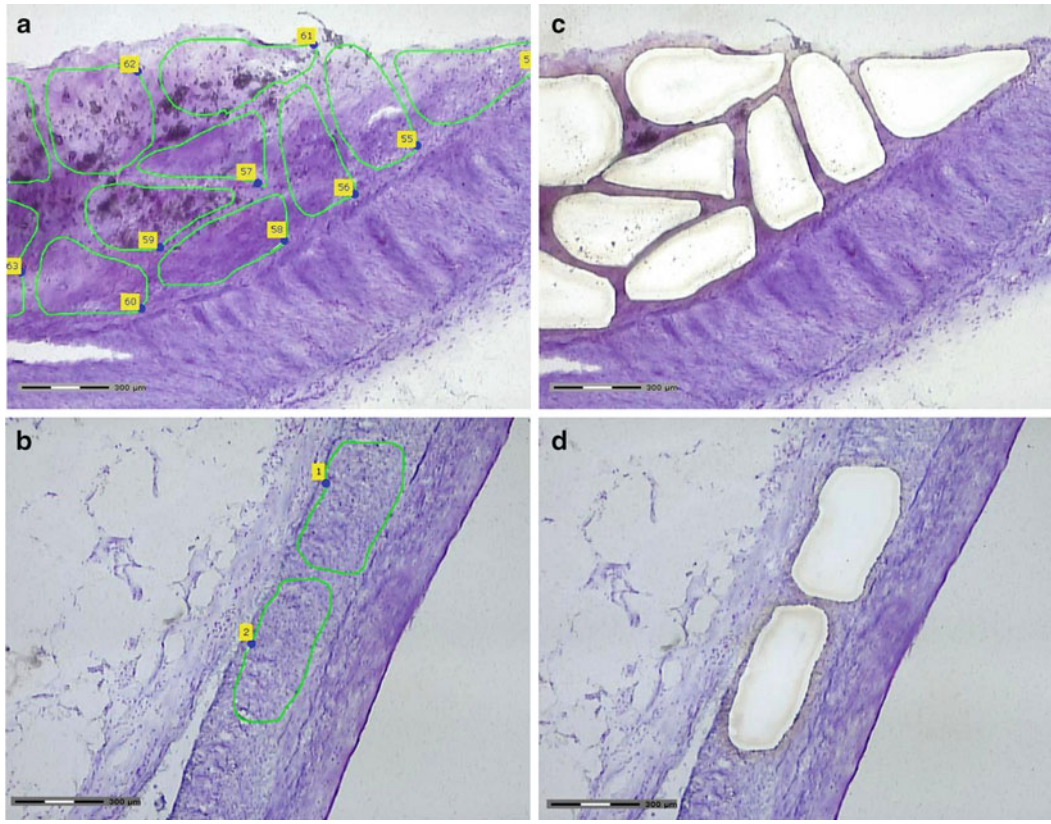

Fig. 2 Isolation of intima and media layers by LMPC. Delimited regions from atherosclerotic coronary intima (**a**) are efficiently isolated by LMD (**b**). Two elements selected within media layer of a preatherosclerotic coronary (**c**) are isolated by LMD, leaving the corresponding blank areas inside the tissue (**d**). From (9) and (10) with permission

cutting function to ensure an efficient isolation of the selected areas. Laser energy power mostly used for arterial regions isolation was 75 % for cutting and 100 % for catapulting. When laser focus and energy have been settled, RoboLPC function can be applied in order to automatically isolate all defined elements (*see* **Note 10**).

3.4 Protein Extraction

1. Collect isolated areas on a 500 µl adhesive cap tube during LMD. Add 100 µl lysis buffer to the tube and incubate with the tube inverted for 5 min on ice. Sonicate three times for 1 min alternated with 1 min on ice. Centrifuge at 12,000 × *g* and place supernatant on a new tube.
2. Place a Protein Desalting Spin Column in a 1.5 ml tube and centrifuge 1 min at 1,500 × *g* to remove retained liquid. Replace the tube for a new one and add the lysate to the column. Centrifuge 2 min at 1,500 × *g* and discard column (*see* **Note 11**).
3. Precipitate the lysate with cold acetone and suspend the pellet in 18 µl labeling buffer.

3.5 Saturation Labeling DIGE (Fig. 3)

1. Check the pH of the lysates to be between 7.8 and 8.2 (ideally 8.0) by spotting 0.2 µl on a pH indicator strip. If necessary, adjust pH by adding either 50 mM NaOH to increase it or 50 mM HCl to lower it (*see* **Note 12**).
2. Rehydrate 24 cm IPG strips pH 4–7 with 450 µl rehydration buffer, 10–24 h previous to sample loading.
3. Create the DIGE internal standard by mixing half of each sample (9 µl).
4. Reduce samples with 1 nmol TCEP (0.5 µl of 2 mM TCEP solution) and the internal standard with the correspondent amount (0.5 µl × number of samples), mix by pipetting, spin down, and incubate 1 h at 37 °C in the dark.
5. Add 2 nmol Cy5 (1 µl of 2 mM resuspended CyDye in DMF) and the correspondent amount to the internal standard (1 µl × number of samples), mix by pipetting, spin down, and incubate 30 min at 37 °C in the dark.
6. Add 1 volume (10.5 µl) of 2× sample buffer.
7. Apply the sample to the rehydrated IPG strips by cup loading. Use the following program for isoelectric focusing (IEF): 200 V for 1 h 30 min, 500 V for 1 h 30 min, 1,000 V for 1 h 30 min, from 1,000 to 8,000 V in 3 h, and 8,000 V until 60,000 V are accumulated.
8. Incubate the IPG strips with 0.01 % DTT in 5 ml of equilibration buffer, 20 min with agitation (*see* **Note 13**).

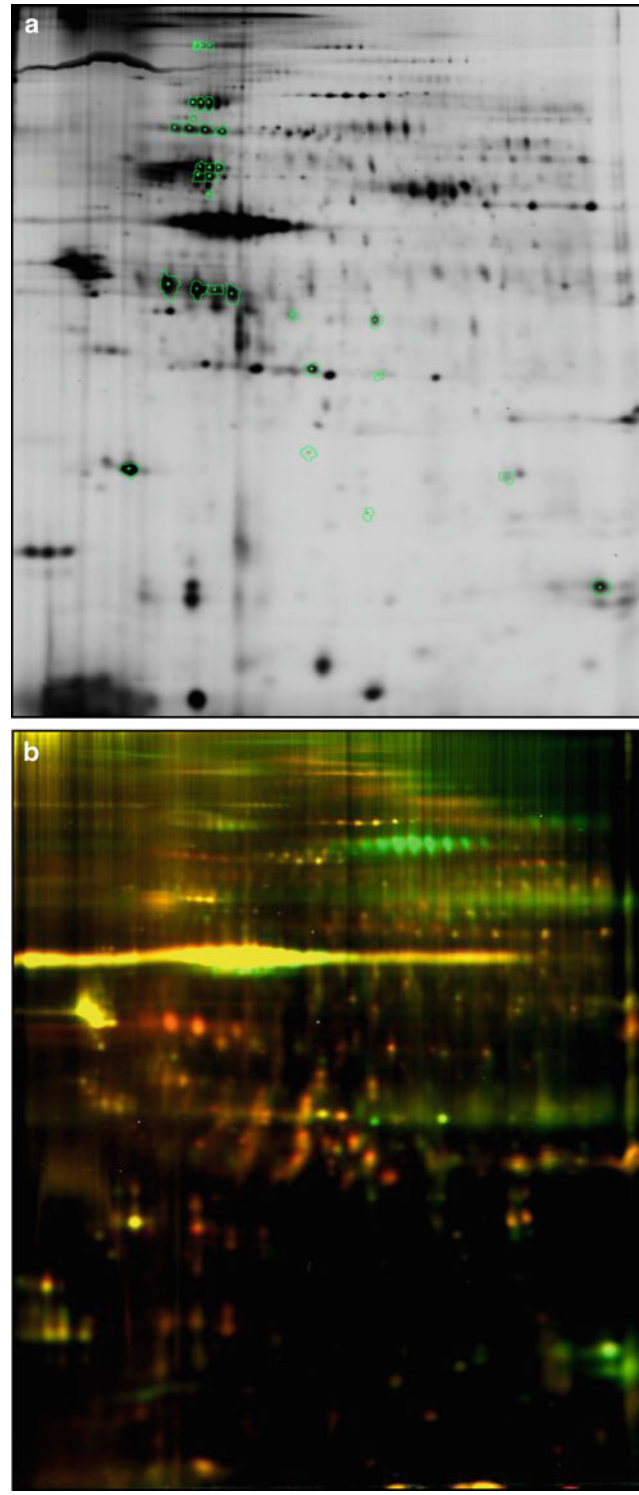

Fig. 3 Representative atherosclerotic coronary 2D-DIGE spot map with differentially expressed spots with respect to healthy coronary and radial artery (p-value <0.05, fold change greater than 2.0 or lower than −2.0, Cy5 fluorochrome gray scale representation, from (10) with permission) (**a**). 2D-DIGE spot map of coronary artery media layer (Cy3/Cy5 merged representation) (**b**)

9. Wash the strips with bidistilled water and place them in 12.5 % polyacrylamide gels. Run the gels on the Ettan Dalt Six device (*see* **Note 14**).
10. Scan the gels on a Typhoon Scanner, firstly at 1,000 ppm to adjust laser power and at 100 ppm to get high-resolution images.
11. Perform differential analysis with DeCyder 2D Software.

3.6 Preparative Gels and MS/MS Identification

1. For the identification of differential spots, use a pool of several LMD protein extracts, if you can collect enough material, or otherwise select a reference proteome, which may be a whole artery extract. Reduce 300 μg of protein with 60 nmol (3 μl of 20 mM TCEP solution) TCEP for 1 h at 37 °C in the dark and label with 120 nmol Cy3 dye (6 μl of 20 mM Cy3 resuspended in DMF) for 30 min at 37 °C in the dark (*see* **Note 15**).
2. Add 1 volume of 2× sample buffer and rehydration buffer to reach final volume of 450 μl. Load sample on a 24 cm IPG strip pH 4–7 (*see* **Note 16**).
3. Subject the strip to the same IEF conditions applied for the comparative analysis, except cup loading. Apply in-gel active rehydration at 50 V for 12 h (*see* **Note 17**).
4. Stain the gels with the Silver Staining Kit. Briefly, fixate them with fixation solution for 30 min, incubate in sensitizing solution for 20 min, rinse three times with bidistilled water for 5 min, incubate in 0.25 % silver nitrate solution for 20 min, and incubate with developing solution until the gel staining is adequate, then stop reaction with stopping solution for 10 min and place the gel in bidistilled water (*see* **Note 18**).
5. Excise the differential spots from the gel and digest them in the DP protein digestion station using the protocol of Shevchenko et al. (11) with minor variations. Briefly, rinse with 50 mM ammonium bicarbonate in 50 % methanol and acetonitrile 70 % and dry in a SpeedVac. Add modified porcine trypsin at a final concentration of 20 ng/μl in 20 mM ammonium bicarbonate and proceed at 37 °C overnight.
6. Extract peptides from gel pieces with 60 % acetonitrile and 0.1 % formic acid.
7. Mix an aliquot of the digestion solution with an aliquot of α-cyano-4-hydroxycinnamic acid in 30 % aqueous acetonitrile, 15 % 2-propanol, and 0.1 % trifluoroacetic acid.
8. Deposit using the thin-layer method onto the MALDI plate (Applied Biosystems) and allow drying at room temperature.
9. Obtain MALDI-MS and MS/MS data in an automated analysis loop with the MALDI TOF/TOF Analyzer (*see* **Note 19**).

4 Notes

1. Five different lysis buffers were analyzed and the composition here reported was the best in terms of protein solubilization and compatibility with IEF analysis (9).
2. Do not place the specimen directly in the liquid nitrogen for embedding, since temperature change after freezing may provoke rupture of the material. Place the material inside an extensive polystyrene recipient for a gradual freezing, which will avoid fragmentation.
3. Oil Red crystallizes very easily, depositing in the slide. To avoid these deposits, place slides upside down in a Petri dish and elevate them from the bottom of the dish using any surface in order to allow tissue contact with the staining solution.
4. Do not dehydrate with graded ethanol series, nor clear with xylene, since this would result in lipids solubilization in such organic solvents, removing them from the tissue. Glycerol gelatin is an aqueous mounting medium and has to be used in this case instead of a nonaqueous mounting medium, like DPX.
5. Prepare DAB solution using gloves, carefully remove it after incubation, and render it inactive with sodium hypochlorite before wasting it in an adequate biohazard container, since it may cause cancer after longtime exposure.
6. Thicker sections would result on greater protein recovery, but thinner sections may be necessary to isolate smaller regions than whole layers. In the latter, 10 μm allow to very specifically isolate them providing enough protein material for subsequent proteomic analysis. PEN membrane slides permit greater regions' catapulting which relies on greater protein recovery and allows checking efficient collection of the catapulted areas by orienting the microscope through the tube cap.
7. Removal of OCT with 4 °C precooled bidistilled water removes less efficiently the compound, since it becomes liquid at RT. Remaining OCT alters or even may impede laser cutting of the tissue.
8. Mayer's hematoxylin provided similar results than Cresyl violet but rendered weaken staining and cannot be solved in pure ethanol, which implies greater proteases activation, which may be important with LMD extended periods. Do not mount and/or cover slides since this would impede LMD. Slides can be stored at -80 °C on a sealed recipient and thawed for LMD isolation when necessary, but this has to be done inside the sealed recipient, since condensation events during thawing may result in tissue hydration that would alter laser cutting. Slides on ice have to be tempered for 5 min before starting LMD.

9. The use of a light diffuser deeply improves visualization of non-mounted specimens, which otherwise are seen in a dark artifact coloring, especially if choosing hematoxylin for staining. Selected regions should not exceed 0.2 mm^2 size, since they may not be well catapulted to the collection tube cap. Although 8 mm^2 provided an adequate protein content for the subsequent 2D-DIGE analysis, greater sample collection may be possible within an adequate LMD timing (<2 h), depending on the efficiency of the procedure and the sample.
10. After applying automatic RoboLPC function, screen the whole slide for areas not well catapulted or that may have fallen from the tube, and use LPC function to manually catapult them back to the tube cap. These mishitting catapulting events are more common at the final steps of the process, where the cap is almost full of material.
11. Tissue staining deeply affects protein solubilization and fluorescent DIGE signal acquisition. Protein Desalting Spin Columns have shown a very efficient removal of remaining staining dye, which in contrast could not be eliminated by acetone precipitation.
12. In order to avoid protein loss during pH checking, pH variation after lysis of the studied sample should be assayed with a preliminary LMD extract and tested during the DIGE experiment with just a few samples to corroborate.
13. In contrast with conventional 2-DE and minimal labeling 2D-DIGE experiments, there is no need to incubate the strips with IAA since cysteine residues will be bound to the saturation labeling CyDye.
14. Run the gels applying constant power, firstly 1 W for all the gels, and when the front dye has clearly entered the gel, apply 20 W per gel for an overday run or 1 W per gel for an overnight one.
15. MS/MS identifications performed in parallel using Cy3 labeled preparative gels and non-labeled ones showed no labeling alterations of the spot pattern in this type of sample, since 29 out of 30 differential spots were identically identified, and the one left could be identified in the non-labeled gel but not in the labeled one.
16. Rehydration buffer for active in-gel rehydration should carry pH 3–10 Pharmalytes, since in this case, protein sample is loaded during rehydration and they facilitate solubilization of all proteins in this pH range, including proteins between 3–4 and 7–10 pH range, which will be excluded with 4–7 pH range Pharmalytes.
17. Cup loading may result on protein precipitation within the cup while working with high protein amounts.

18. Avoid glutaraldehyde in the sensitizing solution since it induces cross-linking of proteins, interfering with mass spectrometry.
19. Analysis of mass data may be performed using the 4000 Series Explorer Software (Applied Biosystems). MALDI-MS and MS/MS data are combined through the GPS Explorer Software to search a nonredundant protein database, such as Swiss-Prot or nrNCBI. Include Cy3 modification in the search engine fixed modifications box.

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