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Laser Microdissection (LMD) for Regional Proteomics

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1 Works for me dx.doi.org/10.17504/protocols.io.brrqm55w

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ABSTRACT

Recent advances in multi-scale interrogation of human tissue, including advanced imaging techniques and the powerful application of large dataset “omics”, have displayed significant promise toward identifying new and specific therapeutic targets, predicting disease progression, and individualizing treatment in participants with acute and chronic kidney disease. Our site, formed by the alliance of Indiana University and Ohio State University, will implement an unbiased tissue interrogation workflow for KPMP human kidney biopsies that integrates largescale 3D tissue imaging for quantitative supervised and unsupervised analysis/cytometry with sub-segmental “omics” data on the same kidney biopsy specimen. The sub-segmental “omics” pipeline will use fluorescence based Laser MicroDissection (LMD) to isolate specific nephron segments and interstitial/other targeted areas, for downstream analysis with transcriptomics and proteomics. The omics analysis will be eventually expanded to include bulk epigenetics. Acting harmoniously with other KPMP sites, our interrogation techniques are expected to facilitate back-mapping of key molecular pathways to the biopsy, which can subsequently identify foci of injury and/or regeneration that can undergo targeted sampling to generate further enriched omics. Therefore, in addition to a significant contribution to the human kidney atlas, our approach will complement other interrogation techniques within KPMP by providing tissue context and increasing spatial resolution for molecular signatures that arise in heterogeneous areas during kidney disease.

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GUIDELINES

The number of peptides detected by mass spectrometer is proportional to the amount of protein in a given sample. This allows for quantitative characterization of protein expression.

Cryosectioning for Regional Proteomics

1 Sections are collected and mounted to LMD slides as specified in the 'Laser microdissection for regional transcriptomics and proteomics' protocol linked below. The LMD slides are then subsequently shipped from IU to OSU for analysis.

Laser microdissection for regional transcriptomics and proteomics
by Michael Eadon

PREVIEW RUN

1.1 All work is performed in a manner that limits RNA contamination and necessitates use of clean disposable gloves and face mask, as well as ensuring the cleanliness of all surfaces (RNAse Away, Ambion, Cat #10328011). This protocol is to be used with kidney tissue preserved in OCT and stored in -80°Celsius.

1.2 1.2 µm Leica PPS-membrane slides (Leica, Cat# 11505268) used for LMD are exposed to UV light (in a tissue culture laminar flow hood with blower off) for 30 minutes, immediately prior to cryosectioning. Slides are stored at room-temp for optimal tissue adherence.

1.3 Cryostat is cooled to -22°Celsius. The work surfaces are cleaned and a new cutting blade is installed.

1.4 A small slide box (cleaned with RNAse Away) is placed inside the cryostat chamber to store slides with freshly cut tissue.

1.5 Specimen in OCT is adhered to a tissue holder and allowed to equilibrate for a few minutes to reach the chamber temperature and strengthen the adhesion between the OCT block and the holder. This process is aided via use of a heat extractor.

1.6 The specimen is cut at the following thickness: a) 12 µm (2 section) – placed in Eppendorf tube for Bulk analysis b) 12 µm (8 sections) – affixed to the specialized Leica LMD slide; one nephrectomy section per slide or two biopsy sections per slide. The slides are stored in - 80°Celsius, with a desiccant cartridge (Bel-Art, Cat# F42046-0000). To prevent any moisture from accumulating inside the slide box, it is further stored in a tightly closed Ziplock® bag. c) 12 µm (1 section) – affixed to a glass slide for Periodic acid-Shiff staining d) 12 µm (2 sections) – miRNA e) 12 µm (6 sections) – For proteomics, tissue is affixed to specialized PENmembrane LMD slides; one nephrectomy section per slide or two biopsy sections per slide. The slides are stored in -80°Celsius, with a desiccant cartridge prior to shipment to OSU. f) 50 µm (2 sections) – stored in tissue culture plate with 4% PFA for 24hr, followed by transfer of tissue into a scintillation vial with 0.25% PFA for long term storage. The tissue in 0.25% PFA is stored at 4°Celsius at all times when not in use. All sections per donor can be placed into the same storage container. Subsequent imaging of 50 um slices is described in section 4.4

1.7 The LMD membrane slide adapter is used to assist with tissue adherence and collection. We prepare 5 to 8 slides.

1.8 Each slide is labeled with a specimen ID, date, and slide number.

1.9 Slides are used within 10 days from the date of cryosectioning.

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1.10 For specimens transferred elsewhere for processing, the tissue is shipped (Mon-Thurs) overnight on dry ice to be received at the destination facility the following business day.

1.11 Add a small amount of OCT to cover the specimen at the end of cryosectioning.

1.12 The Antibody Mix (Ab-Mix) is prepared in 10% BSA (VWR, Cat# 0332-100G) in RNAse-free PBS (VWR, Cat# K812-500ML):

a) **4 µl** OG-Phalloidin (Oregon Green 488, ThermoFisher, Cat# O7466)

b) **1.5 µl** DAPI (ThermoFisher, Cat# 62248)

c) **2 µl** THP antibody (R&D Systems, Cat# AF5144) directly conjugated to Alexa Fluor 555

d) **2 µl** PNA lectin (Vector Laboratories, Cat# FL1071)

e) **3.25 µl** RNAse Inhibitor (ThermoFisher, Cat# AM2696)

f) **87.25 µl** 10% BSA in PBS (to reach a volume of 100 µL)

g) Alternatively: **2 µl** Megalin/LRP2 antibody (Abcam, Cat# ab76969), Host: Rabbit. The Ab-Mix contains either LRP2 or THP antibody (to visualize either proximal tubules or thick ascending limbs, respectively). If LRP2 is used, the protocol requires that Secondary antibody is added (anti-Rabbit AF-555), expanding the "Rapid Stain" protocol for additional **00:05:00** .

1.13 Slide is washed in ice cold (**-20 °C**) 100% Acetone (Sigma-Aldrich, Cat# 270725-1L) for **00:01:00**

1.14 RNAse-free PBS is applied on top of the tissue, 2X **00:00:30** each

1.15 10% BSA in RNAse-free PBS is applied on top of the tissue, 2X **00:00:30** each

1.16 The Ab-Mix (volume depends on the size of the tissue) is applied, for **00:05:00** , in dark

1.17 10% BSA in RNAse-free PBS is applied on top of the tissue, 2X **00:00:30** each

1.18 ***Apply Secondary antibody (if LRP2 is used) for **00:04:00** , followed by 10% BSA in RNAse-free PBS, 2X **00:00:30** each

1.19 The slide is air-dried for **00:05:00** and loaded onto the Leica Laser MicroDissection cutting platform.

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1.20 The micro-dissected tissue segments are collected in the flat cap of an autoclaved **0.5 mL** microcentrifuge tube (ThermoScientific, Cat# AB-0350) appropriate for PCR work, containing **50 µl** of Extraction Buffer from Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Cat# KIT0204).

1.21 Proceed with LMD. Each LMD session should not exceed **02:00:00** . Pre- and post-LMD immunofluorescence images are collected for QC purposes, using Leica DFC7000T camera. In order to obtain 0.5 – 1 ng of RNA, a minimum of 500,000 µm2 area is required. This often necessitates the use of up to 8 x12 µm thick sections to obtain sufficient amount of material for all subsegments of interest. Regions of interest are identified by a trained expert based on staining, morphology and location.

Dissection criteria:

a) Proximal tubule defined by OG-Phalloidin and LRP2
b) Thick ascending loop defined by THP
c) Collecting duct defined by PNA lectin
d) Distal convoluted tubule defined by OG-Phalloidin and morphology and the absence of LRP2, THP, or PNA lectin e) Glomerulus defined by OG-Phalloidin and morphology (collect bowman's capsule in the glomerular dissections)
f) Tubulointerstitium (cortical) defined by neighboring area to a glomerulus
g) Interstitium defined by area between stained tubules

1.22 On each slide, attempt to maximize the dissection of up to 3-4 segments during the 2 hr. The order and frequency of dissection is driven by scarcity, which is specimen dependent. While there is no defined order for all slides, here are some general guidelines on the dissection:
-Please ensure we collect a minimum of 500,000 um^2 for each sub-segment across all slides.
-Remain cognizant of the more "scarce" sub-segments, which include the collecting duct, DCT, and sometimes glomeruli depending on the specimen.
-The scarce sub-segments are collected on more dissections and are often collected with one of the ubiquitous segments (PT or TAL).
-The TI is never collected in an area that has already had tubules dissected. It is paired with glomerular dissections.
-The PT and TAL are never collected together since the megalin and THP Ab's both use the same secondary Ab.
-The interstitium is collected on its own and receives its own dedicated slide because we cut on the opposite side of the basement membrane.

1.23 Special dissections can be collected depending on the tissue contents:
Examples include:
1.If a major artery/large arteriole is identified, collect these cells alone.
2.Collect glomeruli with and without bowman's capsule.
3.Collect areas of injury and inflammation if present.
4.If medulla is present, collect medullary interstitium, or separate cortical/medullary collecting duct, or collect the S3 PT.

1.24 Upon completion of the LMD process, the collecting microcentrifuge tubes are closed and it is ensured that the content moved from the cap to the bottom of the tube (by flicking it vigorously a few times).

1.25 The tubes are centrifuged at 3,000 rcf (Eppendorf, Centrifuge 5424R) for **00:00:30**

1.26 The tubes are incubated in **42 °C** water bath for **00:30:00**

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1.27 The tubes are centrifuged at 3,000 rcf for **00:02:00**

1.28 The supernatant is transferred to a new **0.5 mL** tube and stored in **-80 °C**

1.29 **250 µl** of Conditioned Buffer (CB) is added to each RNA purification column (PC) and incubate for **00:05:00** at room temperature.

1.30 All PCs are centrifuged for **00:01:00** at 16,000 rcf and named conditioned PCs afterwards.

1.31 **50 µl** of 70% Ethanol (provided in the Kit) are added into the tubes with tissue samples. The samples are mixed well by pipetting up and down. Do not vortex. Do not centrifuge.

1.32 The mixture is transferred into conditioned PCs and centrifuged for **00:02:00** at 100 rcf (to bind RNA), quickly followed by **00:00:30** at 16,000 rcf (to remove flow through). This step is repeated if more than 1 tube with tissue samples are available for any given subsegment.

1.33 **100 µl** of Wash Buffer 1 (WB1) is added into the PCs and centrifuged for **00:01:00** at 8,000 rcf.

1.34 **40 µl** of DNAse1 is prepared per each sample (Add **5 µl** of DNAse to **35 µl** of RDD buffer, Qiagen, Cat# 79254). **40 µl** of the mixture is added directly on the membrane of the PC and incubated for **00:15:00** at room temperature.

1.35 **40 µl** WB1 is added onto the membrane of PC, centrifuge for **00:00:15** at 8,000 rcf.

1.36 **100 µl** Wash Buffer 2 (WB2) is added onto the membrane of PC, centrifuge for **00:01:00** at 8,000 rcf.

1.37 **100 µl** WB2 is added onto the membrane of PC, centrifuged for **00:02:00** at 16,000 rcf, immediately followed by centrifugation for **00:01:00** at 16,000 rcf.

1.38 The PC is transferred to a new **0.5 mL** tube.

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1.39 **12 µl** of Elution Buffer (EB) is added onto the membrane and incubated for **00:07:00** at room temperature.
Thus, the final volume of all pooled dissected tissue samples is **12 µl** per subsegment.

1.40 The samples are centrifuged for **00:01:00** at 1,000 rcf (to distribute EB) and then for **00:02:00** at 16,000 rcf.

1.41 **2 µl** are transferred into a fresh tube for Bioanalyzer analysis (to prevent freeze-thaw events).

1.42 All tubes are stored in **-80 °C** until ready for further processing.

1.43 Agilent Bioanalyzer 2100: Eukaryote Total RNA Pico chip.

1.44 Quality control (QC) prior to library prep and sequencing: Quantity > 4 nanograms for bulk. Quantity 0.5 - 1 ng for Subsegment RIN and DV200. DV200 >25% for LMD specimens (optimal > 75%)

1.45 Library prep with Takara SMARTer Stranded Total RNA-Seq pico input v2 for cDNA synthesis. Utilize Option 2 which requires a minimum DV200 of 25% and no fragmentation.

1.46 Addition of Illumina Adapters and Indexes

1.47 Purification of initial RNA-Seq Library Using AMPure Beads

1.48 Depletion of Ribosomal cDNA with ZapR v2 and R-Probes v2

1.49 RNA-Seq Library Amplification

1.50 Purification of final RNA-Seq Library Using AMPure Beads (2 ng/µl cDNA library concentration)

1.51 RNA sequencing 75 bp paired end on Illumina NovoSeq with 30 million reads/sample for bulk and 100 million reads/sample for subsegmental sections

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1.52 Reference RNA used with every sequencing run: Takara Clonetech qPCR Human Reference Total RNA 25 ug Cat #636690. The initial concentration is 1 ug/ul. Our final sequencing concentration for reference RNA is 25 ng/ul. Thus, we have 40 aliquots per package. We use this as a separate sample during library prep. It is run with all of our LMD specimens each time.

1.53 Data analysis with FastQC for sequencing quality check. Assess intergenic and mitochondrial reads. Determine reads attributed to a gene.

1.54 Use Integrative Genomics Viewer (IGV) for alignment.

1.55 edgeR / rbamtools for expression measures for transcripts

1.56 Samples with less than 100,000 genes are removed. The data set is quantile normalized to filter lowly expressed genes.

1.57 Expression is quantified as a ratio of the sub-segment of interest to the average of all other sub-segments and log2-transformed.

1.58 Enrichment analysis to compare gene expression to the set of maker panels specific to each nephron sub-segment based on differential expression.

1.59 Antibody validation: All primary and secondary antibodies are validated either by CLIA or GUDMAP citation, or by internal validation. Internal validation means meeting Human Protein Atlas standards. The minimum level for Ab validation is orthogonal data supporting its specificity.

1.60 Subsegmental collection: All regions of interest are collected based on fluorescent staining, morphology and location. A minimum of 500,000 µm2 area is dissected to obtain sufficient amount of RNA (0.5-1 ng). Each slide is processed (dissected) within at most 2 hours to minimize RNA degradation.

1.61 Images are collected using Leica DFC7000T camera to validate the dissection for inter-operator variability, for archival purposes, training and quality assessment of the performed protocol.

1.62 RNA extraction and Bioanalyzer QC: Minimum RNA quantity required for Bulk is 4 ng and 0.5-1 ng for subsegments. DV200 greater than 25% for all specimens is required (>75% is considered optimal). Our optimum RNA concentration is above 50 pg/µl.

1.63 RNA sequencing: Takara Human Reference RNA (25ng, Cat#636690) is used with each sequencing run.

1.64 Transcriptomic Analysis: Downstream data processing utilizes quantile normalization. Pre-determined marker gene expression will be assessed using enrichment analysis approach.

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1.65 Reference RNA samples are compared across batches. A batch effect within 1 standard deviation of mean expression with R value>0.9 is considered acceptable. Additional normalization is required for higher batch effect score. Each run that deviates from the accepted batch effect will be flagged.

1.66 The Q30 should be >90% for each run.

1.67

The number of peptides detected by mass spectrometer is proportional to the amount of protein in a given sample. This allows for quantitative characterization of protein expression.

Keep slides clean only handle with gloves

1.68 Collect paraffin sections cut 10 µm thickness in mass spec clean container with mass spec H2O (heat water in this container in the tissue water bath…do not heat water in microwave

1.69 After air drying slides, place in desiccator with desiccant “stones” and under vacuum for 7 days

1.70 Prepare all tubes with **13 mL** each of required solutions

1.71 Place slides in 3 changes of octane **00:02:00** each occasionally inverting tube to help with deparaffinization

1.72 Place slides in 3 changes of absolute ethanol **00:02:00** each with occasional gentle inversion

1.73 Place slides in 1 change 90% absolute ethanol **00:02:00** with occasional gentle inversion

1.74 Place slides in 1 change 70% absolute ethanol **00:02:00** with occasional gentle inversion

1.75 Place slides in H2O 2 changes **00:02:00** each with occasional gentle inversion

1.76 Remove slides from H2O and stain with a few drops of hematoxylin for 2-5 seconds then immediately rinse in 2 changes of H2O 2 changes, **00:02:00** each with occasional gentle inversion

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1.77 (NOTE: all of these following steps are done with fresh solutions not used for the deparaffinization) Place slides in 1 change 70% absolute ethanol **00:02:00** with occasional gentle inversion

1.78 Place slides in 95% ethanol for **00:02:00** with occasional gentle inversion

1.79 Place slides in 2 changes of absolute ethanol **00:02:00** each with occasional gentle inversion

1.80 Remove slides and air dry for LMD

1.81 70% Ethanol x **00:02:00** x 2 (done at 4C x 1)

1.82 Place in MS grade water for **00:02:00** x 2

1.83 Stain Slides (Optional) –Stain with Hematoxylin QS

1.84 Place slides in MS grade water for **00:00:30** x 2

1.85 Place slides in 70% ethanol for **00:02:00**

1.86 Place slides in 95% ethanol for **00:02:00**

1.87 Place slides in 100% ethanol # 1 for **00:02:00**

1.88 Place slides in 100% ethanol #2 for **00:02:00**

1.89 Make stock solution of NH3HCO3 1M or 0.5 M store in refrigerator not more than 2 weeks and always look for precipitate meaning time to make fresh stock

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1.90 For single 0.2 mg Rapigest tube to make final of 0.5%, make **200 µl** of NH3HCO3 50 mM NH3HCO3 add it to vial with Rapigest, gently swirl 20-30 seconds

1.91 Keep refrigerated for no more than 2 weeks.

1.92 Use **25 µl** of 0.5% Rapigest to collect each LMPC sample

1.93 Perform LMD on a Leica DFC7000T scope. Collect ~20,000 cells for each sample in under **02:00:00** .

1.94 After LMD collection freeze in PCR tube using dry ice then store at **-80 °C**

1.95 Thaw sample in PCR tubes

1.96 Cut of sides of cap

1.97 Place **0.2 mL** tubes into **1.5 mL** tube with bottom cut off, apply pressure and secure with **10 µl** pipet tip

1.98 Boil sample **00:20:00** then (incubate sample at **60 °C** for two hours – FFPE only)

1.99 Cool and add trypsin (trypsin is 200 ng/µl in 50 mM NH3HCO3) 1:30, briefly centrifuge sample 13,000 RPM, then incubate overnight at **37 °C**

.100 Add formic acid to final volume of 30% and make sure cloudiness seen

.101 Incubate **37 °C** for **00:30:00**

.102 Centrifuge 3X at 15,000 RPM (or so) each time collecting supernatant away from pellet

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.103 Speedvac dry sample and then examine for amber pellet

.104 Add **20 µl** 2% Acetonitrile, 0.1% formic acid, vortex briefly, centrifuge briefly to bring all of solution to bottom of tube

.105 Place in ice cold sonicating water bath for **00:01:00** then briefly centrifuge

.106 Using Nanodrop spectrophotometer, check peptide concentration using 280 absorbance. Use 1.5 µg on single orbitrap run with ~ **05:00:00** gradient.

.107 QA/QC: Determine if protein efficiency of recovery is greater than 50%.

.108 Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis is performed with an Easy-nLC 1000 coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, Waltham, MA).

.109 The LC system configured in a vented format consists of a fused-silica nanospray needle (PicoTip emitter, 75 µm inner diameter) (New Objective, Woburn, MA) packed in-house with 25 cm Magic C18 AQ 100 Å reverse-phase media (Michrom Bioresources, Auburn, CA) and a trap (IntegraFrit Capillary, 100 µm inner diameter) (New Objective, Woburn, MA) containing Magic C18 AQ 200 Å (2 cm).

.110 A measure of 1.5 µg of peptides are loaded onto the column from each sample and separated using a two-mobile-phase system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The chromatographic separation is achieved over a 163-min gradient from 2 to 50% B (5–30% B for **02:30:00** , 30–50% B for **00:10:00** , and 50% B for **00:03:00**) at a flow rate of 300 nl/min.

.111 The mass spectrometer is operated in a data dependent MS/MS mode over the m/z range of 400–1,500. The mass resolution is set to 120,000. The automatic gain control target for the orbitrap is set to 2 × 105 with an injection time of 50 ms.

.112 For MS2, the quadrupole is used for isolation with a window of 1.6 m/z. The cycle time is set to 3 s, and the most abundant ions from the precursor scan with a charge state between 2 and 6 are selected for MS/MS analysis using 27% normalized HCD collision energy and analyzed with an ion trap. The automatic gain control target for the ion trap is set to 1 × 104 with an injection time of 90 ms. Selected ions are dynamically excluded for 20s.

.113 Data analysis is performed using Proteome Discoverer 2.1 (Thermo Scientific).

.114 Trypsin is set as the enzyme with maximum missed cleavages set to 2. The precursor ion tolerance is set to 10 ppm, and the fragment ion tolerance is set to 0.6 Da.

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.115 Variable modifications are set to carbamidomethyl on cysteine residues, and oxidation of methionine residues.

.116 SEQUEST search results are run through Percolator for scoring. Identified peptides are filtered by a 1% peptide-level false discovery rate using q value of 0.01 from Percolator. In addition, false discovery rate at the protein level is calculated, and only the proteins with false discovery rate ≤1% are reported.

.117 The OrbiTrap Fusion mass spectrometer is subjected to a mass calibration every week to ensure the instrument is optimized for obtaining high mass accuracy (< 3 ppm) data.

.118 The mass spectrometer is subjected to full calibration monthly to ensure that not only high mass accuracy is obtained, but to also ensure high sensitivity is obtained.

.119 HPLC function along with the mass spectrometer's mass accuracy and resolution are tested by injecting a five-peptide standard (500 fmol of each peptide) into the LC-MS/MS system.

.120 HPLC retention times for each peptide should not deviate more than one minute from historic norms, mass accuracy should be less than 3 ppm, and mass resolution should be better than 120,000 at m/z 200.

.121 This five-peptide mixture is run on a daily basis and run intermittently during the analysis of large numbers of samples to verify the instrument's performance at the beginning, middle, and end of large sample sets.

.122 The full LC-MS/MS data collection mode is tested after full instrument calibrations and before long sample sets by injection of 500 nanograms of a trypsin-digested yeast lysate (Promega).

.123 A minimum of 3000 proteins should be identified (less than 1% false discovery rate) after automated protein database searching.

.124 If peptide retention times, mass accuracy, or protein identification numbers do not meet defined criteria, the LC-MS/MS system is evaluated and steps are taken (e.g. cleaning of the mass spectrometer) to rectify the problem.

.125 Enrichment analysis to compare protein abundance to the set of maker panels specific to each nephron sub-segment.

Preparation of Frozen Sections for LMD 12m 30s

2 70% Ethanol **00:02:00** X 2 (done at **4 °C**) 2m

3 Place slides in MS grad water for **00:02:00** X 2. Fresh MS grade water should be used with each wash. 2m

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4 Stain slides with Hematoxylin QS (optional)

4.1 (If staining with Hematoxylin) Place slide in MS grade water for **00:00:30** X 2 30s

5 Place slides in 70% ethanol for **00:02:00** 2m

6 Place slides in 95% ethanol for **00:02:00** 2m

7 Place slides in 100% ethanol #1 for **00:02:00** 2m

8 Place slides in 100% ethanol #2 for **00:02:00** 2m

Collection of LMD Tissue 12m 30s

9 Make stock solution of NH3HCO3 1M or 0.5 M

Store in refrigerator for no more than 2 weeks and always look for precipitate. Presence of precipitate means it is time to make fresh stock.

10 For single 0.2 mg Rapigest tube to make final of 0.5%, make **200 µl** of 50 mM NH3HCO3 add it to vial with Rapigest. Gently swirl **00:00:20** - **00:00:30** . 50s

Keep the solution refrigerated for no more than 2 weeks.

11 Use **25 µl** of 0.5% Rapigest to collect each LMPC sample.

12 Perform LMD on a Leica DFC7000T scope. Collect ~20,000 cells for each sample in under **02:00:00** 2h

13 After LMD collection, freeze in PCR tube using dry ice and then store at **-80 °C**

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Trypsin Digestion 12m 30s

14 Thaw samples in PCR tubes

15 Cut off the sides of the tube cap

16 Place **0.2 mL** tubes into **1.5 mL** tubes with the bottoms cut off. Apply pressure and secure with **10 µl** pipette tip.

17 Boil sample **00:20:00** 20m

17.1 (Only if FFPE) Incubate sample at **60 °C** for **02:00:00** 2h

18 Cool and add trypsin (trypsin is 200 ng/µl in 50 mM NH3HCO3) 1:30, briefly centrifuge sample **13000 rpm** , then incubate overnight at **37 °C**

The amount of trypsin added is 1/30th the amount to total protein in the sample.

19 Add formic acid to final volume of 30% and make sure cloudiness is visualized

20 Incubate at **37 °C** for **00:30:00** 30m

21 Centrifuge for **00:10:00** at **15000 rpm** and collect the supernatant without disturbing the pellet. Repeat two more times for a total of three centrifugations. 10m

22 Speedvac dry the sample and confirm presence of an amber pellet

23 Add **20 µl** total of 2% acetonitrile and 0.1% formic acid mixture

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24 Vortex briefly, centrifuge briefly to bring all of the solution to the bottom of the tube.

25 Place the tube in an ice cold sonicating water bath for **00:01:00** then briefly centrifuge
1m

26 Using Nanodrop spectrophotometer, check the peptide concentration using 280 absorbance. Use **1.5 µg** on single orbitrap run with ~ **05:00:00** gradient
5h

27 QA/QC: Determine if protein efficiency of recovery is > 50%

HLPC and Mass Spectrometry 12m 30s

28 Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis is performed with an Easy-nLC 1000 coupled to an Orbitrap Eclipse mass spectrometer (Thermo Scientific, Waltham, MA).

Orbitrap Eclipse
Mass Spectrometer
Thermo Scientific **00-0000**

29 The LC system configured in a vented format consists of a fused-silica nanospray needle (PicoTip emitter, 75 µm inner diameter) (New Objective, Woburn, MA) packed in-house with 25 cm Magic C18 AQ 100 Å reverse-phase media (Michrom Bioresources, Auburn, CA) and a trap (IntegraFrit Capillary, 100 µm inner diameter) (New Objective, Woburn, MA) containing Magic C18 AQ 200 Å (2 cm).

30 A measure of **1.5 µg** of peptides are loaded onto the column from each sample and separated using a two-mobile-phase system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The chromatographic separation is achieved over a 163-min gradient from 2 to 50% B (5–30% B for **02:30:00** , 30–50% B for **00:10:00** , and 50% B for **00:03:00**) at a flow rate of 300 nl/min
2h 43m

31 The mass spectrometer is operated in a data dependent MS/MS mode over the m/z range of 400–1,500. The mass resolution is set to 120,000. The automatic gain control target for the orbitrap is set to 2 × 105 with an injection time of 50 ms.

32 For MS2, the quadrupole is used for isolation with a window of 1.6 m/z. The cycle time is set to 3 s, and the most abundant ions from the precursor scan with a charge state between 2 and 6 are selected for MS/MS analysis using 27% normalized HCD collision energy and analyzed with an ion trap. The automatic gain control target for the ion trap is set to 1 × 104 with an injection time of 90 ms. Selected ions are dynamically excluded for 20s.

33 Data analysis is performed using Proteome Discoverer 2.2 (Thermo Scientific).

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34 Trypsin is set as the enzyme with maximum missed cleavages set to 2. The precursor ion tolerance is set to 10 ppm, and the fragment ion tolerance is set to 0.6 Da.

35 Variable modifications are set to carbamidomethyl on cysteine residues, and oxidation of methionine residues.

36 SEQUEST search results are run through Percolator for scoring. Identified peptides are filtered by a 1% peptide-level false discovery rate using q value of 0.01 from Percolator. In addition, false discovery rate at the protein level is calculated, and only the proteins with false discovery rate ≤1% are reported.

Proteomics QC and OrbiTrap Eclipse 12m 30s

37 The OrbiTrap Eclipse mass spectrometer is subjected to a mass calibration every week to ensure the instrument is optimized for obtaining high mass accuracy (< 3 ppm) data.

38 The mass spectrometer is subjected to full calibration monthly to ensure that not only high mass accuracy is obtained, but to also ensure high sensitivity is obtained.

39 HPLC function along with the mass spectrometer's mass accuracy and resolution are tested by injecting a five-peptide standard (500 fmol of each peptide) into the LC-MS/MS system.

40 HPLC retention times for each peptide should not deviate more than one minute from historic norms, mass accuracy should be less than 3 ppm, and mass resolution should be better than 120,000 at m/z 200.

41 This five-peptide mixture is run on a daily basis and run intermittently during the analysis of large numbers of samples to verify the instrument's performance at the beginning, middle, and end of large sample sets.

42 The full LC-MS/MS data collection mode is tested after full instrument calibrations and before long sample sets by injection of **200 ng** of a trypsin-digested yeast lysate (Promega).

43 A minimum of 6000 proteins should be detected for digest (less than 1% false discovery rate) after automated protein database searching.

44 If peptide retention times, mass accuracy, or protein identification numbers do not meet defined criteria, the LC-MS/MS system is evaluated and steps are taken (e.g. cleaning of the mass spectrometer) to rectify the problem.

45 Enrichment analysis to compare protein abundance to the set of maker panels specific to each nephron sub-segment.

Cutting & Deparaffinization of FFPE Sections (FFPE Only, Protocol not active in KPMP) 12m 30s

46 Collect paraffin sections cut 10 µm thickness in mass spec clean container with mass spec H2O (heat water in this container in the tissue water bath…do not heat water in microwave.

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47 After air drying slides, place in desiccator with desiccant “stones” and under vacuum for 7 days

48 Prepare all tubes with **13 mL** each of required solutions

49 Place slides in 3 changes of octane **00:02:00** each occasionally inverting tube to help with deparaffinization 2m

50 Place slides in 3 changes of absolute ethanol **00:02:00** each with occasional gentle inversion 2m

51 Place slides in 1 change 90% absolute ethanol **00:02:00** with occasional gentle inversion 2m

52 Place slides in 1 change 70% absolute ethanol **00:02:00** with occasional gentle inversion 2m

53 Place slides in H2O 2 changes **00:02:00** each with occasional gentle inversion 2m

54 Remove slides from H2O and stain with a few drops of hematoxylin for 2-5 seconds then immediately rinse in 2 changes of H2O 2 changes, **00:02:00** each with occasional gentle inversion 2m

NOTE: All of the following steps are done with fresh solutions not used for the deparaffinization

55 Place slides in 1 change 70% absolute ethanol **00:02:00** with occasional gentle inversion 2m

56 Place slides in 95% ethanol for **00:02:00** with occasional gentle inversion 2m

57 Place slides in 2 changes of absolute ethanol **00:02:00** each with occasional gentle inversion 2m

58 Remove slides and air dry for LMD

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