SOP

FFPE-TRYPSIN-WORKFLOW FOR MALDI IMAGING

1. CHEMICALS AND MATERIAL
   - Formalin-fixed and paraffin embedded tissue (FFPE-tissue)
   - Ethanol (MS-grade)
   - Methanol MeOH (MS-grade)
   - Xylol (MS-grade)
   - Acetonitrile ACN (MS-grade)
   - 100 mM Ammonium bicarbonate AMBIC (Sigma Aldrich)
   - MilliQ Water
   - Acetic acid (99.5%)
   - Trifluorocacetic acid (TFA)
   - Matrix (CHCA or DHB)
   - Trypsin Gold (Promega)
   - Peptid Standard (Bruker)
   - Crushed Ice

2. EQUIPMENT
   - ITO-Slide (Bruker) coated additional with Poly-L-lysine and IGEPAL
   - Eppendorf Safe-lock microcentrifuge tubes (1.5 ml or 0.5 ml)
   - Vortex mixer
   - Pipettes and pipette tips
   - Glass cuvette
   - Microtom (Leica)
   - Equipment for sectioning (brushs, tweezers, microtome blade)
   - White paint marker (Edding 780, 0.8 mm)
   - Permament, solve resistant Marker (Secureline)
   - Heating cabinet
   - Water bath
   - SunPrep (SunChrom)
   - SunCollect (SunChrom)
   - SunDigest (SunChrom)
   - Slide Scanner (Hamamatsu, NanozoomerSQ)
   - UltrafleXtreme (Bruker)
   - Slide Adapter II (Bruker)

3. PRELIMINARY

   Acetic acid 50 mM
   add 115 µl Acetic acid (99.5%) to 40 ml Milli-Q

   AMBIC (Ammonium bicarbonate NH₄HCO₃)
   100 mM Stock solution (394 mg in 50 ml Milli-Q)
   50 mM => for trypsin working solution
   10 mM => for ph-conditioning
Citric Acid Monohydrate ($C_6H_8O_7$·$H_2O$) 10 mM pH 6
1.05 g in 500 ml Milli-Q => for Antigen-Retrieval

Trypsin-Aliquot
100 µg lyophilized trypsin powder
add 200 µl Acetic acid (50 mM) = 0,5 µg/µl
=> 5 Aliquots à 40 µl (store at -80°C)
Trypsin working solution (100 ng/µl) – store on crushed ice until use
40 µl Trypsin (0,5 µg/µl)
+ 160 µl 50 mM AMBIC
+ 10 µl ACN

Peptide standard aliquot
peptide standard II stored at -20°C until dissolving
0.1% TFA: add 1 µl TFA to 999 µl MilliQ water
Dissolve the peptide standard in 125 µl 0.1% TFA solvent
vortex for several seconds
aliquot the dissolved peptide standard in 5 µl
store at -20°C until use

Matrixsolutions (fresh or storage for maximum 1 week at room temperature in dark)
CHCA (10 mg/ml in 60% ACN + 0.2% TFA)
   10 mg CHCA + 600 µl ACN + 400 µl H₂O + 2 µl TFA
DHB (30 mg/ml in 50% MeOH + 0.2% TFA)
   30 mg DHB + 500 µl MeOH + 500 µl H₂O + 2 µl TFA

4. Operating Procedure
- Sectioning FFPE tissue (Leica microtome) 5 µm
  let dry the slides at room temperature
  for later use: store at room temperature

- pre melt the section
  1 hours at 60°C in the heating chamber or overnight at 37°C

- deparaffinization and rehydration (with SunPrep)
  (each step 220s, 4 dips all 5 seconds)
Xylol I → Xylol II → Xylol III → drying (compressed air)
→ Ethanol 100% → Ethanol 95% → Ethanol 70% → drying (compressed air)
→ 10mM AMBIC → H₂O → H₂O → drying (compressed air)

- antigen retrieval
  Slide in glass cuvette filled with Citric acid (10 mM, pH6) or Tris-HCl (10 mM, pH9)
  place in the cold water bath
  heat the water bath to 95°C, cook the slide 30 minutes at 95°C (both steps take approximately 1h)
• **pH-conditioning (with SunPrep)**
  (each step 220s, 4 dips all 5 seconds)

  10mM AMBIC → 10mM AMBIC → H₂O → drying (compressed air)

• Teach marks (white marker)

• **Overview Scan (NanoZoomer SQ)**
  change storage location and file name, manual adjustment of the scanning area (tissue and tech marks),
  check focus points manual, magnification 20x

• **Trypsin deposition SunCollect**
  pre-cool SunCollect syringe (250 µl) filled with AMBIC (50 mM) on crushed ice

  Trypsin working solution **(ON ICE):**
  40 µl Trypsin Aliquot
  + 160 µl AMBIC (50 mM)
  + 10 µl ACN

  **Trypsin spray protocol** (e.g. Trypsin area_3.cfg)
  8 layers á 10 µl/min
  Speed x = low, 7
  Speed y = medium, 1
  Line distance in y = 2 mm
  Z position = 29 mm, z offset = 0

• **Tryptic adigestion with SunDigest**
  **SMART or BASIC mode**

  **SMART mode** (C:\ProgramFiles\SunDigest1.18\Smartmode_methods\smartmode_fan10.txt)
  No. of Steps per Cycle: 2; Cycle restriction: YES (1); cooldown: YES (10 min)

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Fan Speed</th>
<th>Base Temp</th>
<th>Cover Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>900 s</td>
<td>0 %</td>
<td>50°C</td>
<td>45°C</td>
</tr>
<tr>
<td>Step 2</td>
<td>6300 s</td>
<td>10%</td>
<td>50°C</td>
<td>45°C</td>
</tr>
</tbody>
</table>

  **BASIC mode**
  Humidity: 95%, Temperature: 50°C, Time: 2:00:00, Cooldown ❌
  No. of Steps per Cycle: 2; Cycle restriction: YES (1); cooldown: YES (10 min)

• **Matrix deposition with SunCollect**
  **CHCA or DHB**
  fill the matrix solution in the SunCollect syringe (2.5 ml), spray the matrix with the spraying device
  (SunCollect) according the following protocols or create a new one
**CHCA spray protocol** (e.g. HCCA area_4.cfg)
3 layers => layer 1: 10 µl/min; layer 2/3: 35 µl/min
Speed x = low, 3
Speed y = medium, 1
Line distance in y = 2 mm
Z position = 29 mm, z offset = 0

**DHB spray protocol (30 mg/ml)** (e.g. DHB area_4.cfg)
5 layers => layer 1: 10 µl/min; layer 2: 35 µl/min
Speed x = low, 3
Speed y = medium, 1
Line distance in y = 2 mm
Z position = 29 mm, z offset = 0

- Calibration with peptide standard
  5 µl Peptide standard Aliquot (stored at -20°C)
  + 5 µl Matrix solution
  spot 2x 1 µl on the ITO Slide next to the matrix-tissue-areal

- Transfer the Slide adapter with the prepared slide to the UltraflexXtreme
  calibrate the flexControl method
  select the AutoXecute method (check the flexControl method)
  open flexImaging, teach the slide (white marks) – check teaching on several points at the tissue
  define measurement region (tissue region), define additionally small matrix
  start the measurement

- After measurement:
  histological staining (hematoxylin/eosin, PAS or other)
  interpretation with SCiLS software

5. **ADDITIONAL**

**Coordinates for spray protocol** (individual adjustment is possible)

<table>
<thead>
<tr>
<th></th>
<th>X1</th>
<th>Y1</th>
<th>X2</th>
<th>Y2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area 1</td>
<td>8,37</td>
<td>2,55</td>
<td>64,25</td>
<td>32,55</td>
</tr>
<tr>
<td>Area 2</td>
<td>20,37</td>
<td>2,55</td>
<td>52,25</td>
<td>32,55</td>
</tr>
<tr>
<td>Area 3</td>
<td>20,37</td>
<td>12,55</td>
<td>52,25</td>
<td>32,55</td>
</tr>
<tr>
<td>Area 4</td>
<td>8,37</td>
<td>12,55</td>
<td>64,25</td>
<td>32,55</td>
</tr>
</tbody>
</table>
### Schematic Overview FFPE-Trypsin-Workflow

#### FFPE-Trypsin-Workflow

**Paraffin section 5 µm**

<table>
<thead>
<tr>
<th>Step</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-melt</strong></td>
<td>60°C, 1h (or overnight 37°C)</td>
</tr>
<tr>
<td><strong>Deparaffinization</strong></td>
<td>220 seconds&lt;br&gt;↓ Xylo 1&lt;br&gt;↓ Xylo 2&lt;br&gt;↓ Xylo 3&lt;br&gt;↓ dry</td>
</tr>
<tr>
<td><strong>Rehydration</strong></td>
<td>↓ Ethanol 100&lt;br&gt;↓ Ethanol 95%&lt;br&gt;↓ Ethanol 70%&lt;br&gt;↓ dry</td>
</tr>
<tr>
<td><strong>pH-conditioning 1</strong></td>
<td>↓ AMBIC 10mM&lt;br&gt;↓ H₂O&lt;br&gt;↓ H₂O&lt;br&gt;↓ dry</td>
</tr>
<tr>
<td><strong>Antigen-Retrieval</strong></td>
<td>30 min. 95°C water bath&lt;br&gt;cold start – at reach 95°C – 30 min. cook&lt;br&gt;cuvette filled with: Citric acid 10mM pH6</td>
</tr>
<tr>
<td><strong>pH- conditioning 2</strong></td>
<td>220 seconds&lt;br&gt;↓ AMBIC 10mM&lt;br&gt;↓ AMBIC 10mM&lt;br&gt;↓ H₂O&lt;br&gt;↓ dry</td>
</tr>
<tr>
<td><strong>Teach Marks, Scan</strong></td>
<td>SunCollect syringe filled with AMBIC 50mM precool on Ice</td>
</tr>
<tr>
<td><strong>Trypsin</strong></td>
<td>Trypsin deposit with SunCollect&lt;br&gt;Trypsin working solution: 40 µl Trypsin Aliquot&lt;br&gt;on Ice&lt;br&gt;+ 160 µl AMBIC 50 mM&lt;br&gt;+ 10 µl ACN</td>
</tr>
<tr>
<td><strong>Digestion</strong></td>
<td>tryptic digestion with SunDigest&lt;br&gt;SMART or BASIC mode</td>
</tr>
<tr>
<td><strong>Matrix</strong></td>
<td>Matrix deposit with SunCollect</td>
</tr>
<tr>
<td><strong>Peptide standard for calibration on matrix-free areal</strong></td>
<td></td>
</tr>
<tr>
<td><strong>MSI measurement</strong></td>
<td>calibration, matrix-spectra, tissue region</td>
</tr>
<tr>
<td><strong>Histologische Färbung</strong></td>
<td>hematoxylin/eosin, PAS or other</td>
</tr>
</tbody>
</table>