Clinical tissue analysis: overview of workflows and diagnostic "blind spots"

1. Clinical cases

2. Overview of clinical diagnostic workflows

3. Background on mass spectrometry and current clinical applications

4. Diagnostic “Blind spots”
**Clinical Case 1**

- 38 year-old woman with no significant past medical history brought to the ED after being found unconscious at work.

- MRI revealed a large non-enhancing lesion involving the left temporal lobe and extending into the insula with significant mass effect on the brainstem.

- Underwent an awake left temporal craniotomy for tumor resection and decompression of the brainstem.

**Diagnostics**

<table>
<thead>
<tr>
<th>H&amp;E</th>
<th>MIB-1 (&lt;3%)</th>
</tr>
</thead>
</table>

- Cytogenetics: no clinically significant detection of 1p and 19q co-deletion

- Diagnosis: MIXED GLIOMA (OLIGOASTROCYTOMA) without definite anaplastic features, WHO Grade II/IV; IHC: Positive - MIB-1 (qualitative index <3%), GFAP, OLIG2

**Treatment:** Treated with radiation therapy and temozolomide. Serial imaging showed stable disease (~4 years)

---

**Clinical Case 1 continued...5 years later**

<table>
<thead>
<tr>
<th>IDH1</th>
<th>MIB-1 (21.7%)</th>
</tr>
</thead>
</table>

- Molecular/IHC: MGMT- promoter methylated; IDH1(R132H) positive

- Diagnosis: RECURRENT/RESIDUAL MIXED GLIOMA (OLIGOASTROCYTOMA) with anaplastic features, probably classifiable as W.H.O. Grade III.
Clinical Case 1 continued...1 year later

• Severe headache
• Rapid neurological decline

RECURRENT/RESIDUAL GLIOMA with features of GLIOBLASTOMA (IDH1 R132H mutated; MGMT promoter methylated; aCGH summary: single copy loss of CDKN2A, PTEN and RB1; partial deletion of 22q), W.H.O. Grade IV, with evidence of treatment effect.

Clinical Case 2

• 65 year old woman with a history of cardiac sarcoidosis, status post heart transplant. Found to have an enlarging pericardial mass
Clinical case 3

- 42 year old woman treated for many years with an intrathecal morphine delivery device.
- Presents with significant and progressive neurologic deficits.
- MRI demonstrates mass and local inflammation near the intrathecal catheter tip.
- Mass emergently resected.

Three clinical cases and tissue based diagnostic journeys...

...can we do better?
Introduction to clinical diagnostic workflows

Clinical Diagnostics
Pathology Department (BWH)

Anatomic Pathology (AP)
Clinical Pathology (CP) aka Lab Medicine

Images care of Wikipedia
Anatomic Pathology (AP)

Modern Anatomic Pathology (AP):
- Surgical Pathology
- Cytopathology
- Autopsy Pathology
- Forensic Pathology

AP at BWH:
- ~80,000 specimens/year
- ~200-250 specimens/day
- >50% related to cancer

Cancer surgery: a “durable” option

Frozen section Procedure

Image courtesy of Computational Neurosciences Outcome Center BWH Website: Cushing’s 2000th verified brain tumor operation, 1931

Image courtesy of Lab Tests Online: Anatomic Pathology
Frozen Section Procedure: workflow

1. Tissue Excision
2. Chuck Placement
3. Freezing
4. Trimming
5. Pathologist consultation
6. Staining
7. Thaw-mount
8. Sectioning

Frozen section procedure

- **Benefits**
  - “Time tested”
  - Universally available
  - Provides microscopic/cellular characterization

- **Limitations**
  - Turnaround time
  - Frozen section “artifact”
  - Intra-user variability
  - Largely limited to H&E staining

Published in the December 2, 1905, issue of *JAMA*. 

MSACL Connect: Imaging 101: Clinical Studies using Imaging MS ©2020 Sankha Basu, Michelle Reyzer, Christina Ferreira
Permanent section

Tissue Excision → Fixation → Grossing → Processing

Staining ← Mounting ← Sectioning ← Embedding

Process can take several days

Tissue stains

H&E

IHC

Special Stains

Each additional stain can add extra days to result time
Clinical Pathology (CP)

Modern Clinical Pathology (CP):
- Chemistry
- Hematology
- Microbiology
- Molecular Pathology
- Transfusion Medicine

CP at BWH:
- ~7 million results/year
- ~20,000 tests/day
What is a Mass Spectrometer?

- An instrument that detects and measures molecules based on their mass-to-charge ratio (m/z)

“A molecular scale”
Mass Spec: “An oldie but a goodie”

Mass Spec: “An oldie but a goodie”

Modern MS: many shapes and sizes

LC-MS/MS (BWH Clinical Chemistry)

MALDI-TOF MS (BWH Clinical Microbiology)
Common mass analyzers

Quadrupole

Time of flight (ToF)

Linear and reflector TOF MS

Ion Trap

Fourier Transform Ion Cyclotron Resonance (FT-ICR)

The right tool for the job
Current clinical MS applications

Application 1:
LC-MS/MS in clinical chemistry
Opioid Epidemic

Common Opiates/Opioids

HEROIN | CODEINE | HYDROCODONE | OXYCODONE | BUPRENORPHINE

6-MAM | MORPHINE | HYDROMORPHONE | OXYMORPHONE | FENTANYL

Immunoassay | HPLC

METHADONE | EDDP
Similar analytes are difficult to separate
But suppose there was a way to distinguish co-eluting analytes

Oxycodone

Oxymorphone

....enter Mass Spec

Tandem MS

Quantifier

Qualifier
Importance of internal standards

Analytical concerns:
- Stability during extraction
- Stability in autosampler
- Ion suppression
- Ionization efficiency
- Gradient elution solvent effects

Sample Collection → Processing → Separation → Measurement

Reversed-phase Liquid Chromatography (LC)
Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS)

Stable isotope Internal standard

Sample chromatogram

Correlation coefficient: r = 0.999456, r^2 = 0.998912
Calibration curve: 𝑦 = 0.533637 * x + 0.0296476
Response type: Internal Std (Ref 19), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None
Urine Toxicology Screen

- Automated immunoassay performed on urine samples to test for a number of different drug classes.
- Pros: Fast turnaround time (TAT): Performed 24 hours/day, 7 days/week, (result in hours)
- Cons: Sensitivity: higher cutoffs compared with LC-MS/MS based methods
  - Specificity: is susceptible to false positive and false negative due to cross-reactivity

<table>
<thead>
<tr>
<th>Cutoff in urine (ng/mL)</th>
<th>Drugs that can cause false positive results</th>
<th>Drugs that can cause false negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPHETAMINES 1000</td>
<td>Trazodone</td>
<td>1-methylamphetamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-amphetamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phentermine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pseudoephedrine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phenylpropanolamine</td>
</tr>
<tr>
<td>BENZODIAZEPINES 100</td>
<td>Oxaprozin</td>
<td>Alprazolam</td>
</tr>
<tr>
<td></td>
<td>Orphenadrine</td>
<td>Lorazepam</td>
</tr>
<tr>
<td>OPIATES 300</td>
<td>Buprenorphine (very high levels)</td>
<td>Oxycodeine</td>
</tr>
<tr>
<td></td>
<td>Naltrexone</td>
<td>Oxymorphone</td>
</tr>
<tr>
<td></td>
<td>Quinidines</td>
<td>Meperidine</td>
</tr>
<tr>
<td>METHADONE 300</td>
<td>Cyamemazine</td>
<td>Norcodeine</td>
</tr>
<tr>
<td></td>
<td>Quetiapine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tramadol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methotrexprazine</td>
<td></td>
</tr>
<tr>
<td>OXYCODONE 300</td>
<td>Labetalol T</td>
<td>Norfentanyl</td>
</tr>
<tr>
<td>FENTANYL 4</td>
<td>Trazodone</td>
<td>Opiates</td>
</tr>
<tr>
<td>CANNABINOIDS 50</td>
<td>Norfentanyl</td>
<td></td>
</tr>
<tr>
<td>COCAINE (metabolite) 150</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comparison of Immunoassay to LC-MS/MS

<table>
<thead>
<tr>
<th>Immunoassay</th>
<th>LC-MS/MS</th>
<th>Drug</th>
<th>Cutoff (ng/mL)</th>
<th>Drug</th>
<th>Cutoff (ng/mL)</th>
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<tbody>
<tr>
<td>Opiates 300</td>
<td></td>
<td>Codeine</td>
<td>25</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Hydrocodone</td>
<td>25</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Morphine</td>
<td>25</td>
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<tr>
<td></td>
<td></td>
<td>3-beta-morphine-glucuronide</td>
<td>25</td>
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<tr>
<td></td>
<td></td>
<td>6-beta-morphine-glucuronide</td>
<td>25</td>
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<tr>
<td></td>
<td></td>
<td>Hydromorphone</td>
<td>25</td>
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<td></td>
<td></td>
<td>Hydroxycodone</td>
<td>25</td>
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<td></td>
<td></td>
<td>Oxycodeine</td>
<td>25</td>
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<td></td>
<td>Norfentanyl</td>
<td>25</td>
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<tr>
<td></td>
<td></td>
<td>Norfentanyl-glucuronide</td>
<td>25</td>
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<tr>
<td></td>
<td></td>
<td>Noroxycodone</td>
<td>25</td>
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<td></td>
<td>Oxymorphone</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fentanyl</td>
<td>1</td>
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<td></td>
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<td>Norfentanyl</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td>Methadone</td>
<td>5</td>
<td></td>
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<td></td>
<td></td>
<td>Methadone-glucuronide</td>
<td>5</td>
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<tr>
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<td>EDDP (methadone metabolite)</td>
<td>5</td>
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<td>Tramadol</td>
<td>200</td>
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<tr>
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<td></td>
<td>Tramadol-glucuronide</td>
<td>200</td>
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<td></td>
<td>Norfentanyl</td>
<td>5</td>
<td></td>
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<tr>
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<td></td>
<td>Norfentanyl-glucuronide</td>
<td>5</td>
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<td></td>
<td>Naloxone</td>
<td>100</td>
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<tr>
<td></td>
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<td>Hydromorphone</td>
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<td></td>
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<td></td>
<td></td>
<td>Hydrocodone</td>
<td>25</td>
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<td></td>
<td>Noroxycodone</td>
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<td></td>
<td>Oxymorphone</td>
<td>25</td>
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</tbody>
</table>
Current clinical MS applications

Application 2: MALDI TOF for microbial identification

Application 2: Improve clinical TAT for bacterial ID

• Clinical problem: Bacteremic patients are often left on suboptimal antibiotic regimens until speciation of infectious organism can be made.

  Many can be identified on the same day based on morphology & rapid spot tests.

  Most cannot be ID’d same day due to phenotypic similarities.
Solution: MALDI-TOF MS for bacterial identification

Two FDA approved instruments using in microbiology

VITEK MS (Biomerieux, Shimadzu)  Microflex Biotyper (Bruker Daltonics)

BWH MGH

CHB
Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI TOF) Mass Spectrometry for microbial identification

Chemical matrix (0.5 µl α-cyano-4-hydroxycinnamic acid)

Target Slide 48 wells

MALDI-TOF mass spectra

*Escherichia coli*

*Bacillus subtilis*

*Candida albicans*

*Aspergillus fumigatus*
Spectrum is transformed into list of peak (bins)

Interprets spectrum as sum of bins

Bins for unknown isolate

<table>
<thead>
<tr>
<th>Bin</th>
<th>Bin 1</th>
<th>Bin 2</th>
<th>Bin 3</th>
<th>Bin 4</th>
<th>Bin 5</th>
<th>Bin 1300</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>0</td>
<td>+20</td>
<td>0</td>
<td>-15</td>
<td>+4</td>
<td>-3</td>
</tr>
<tr>
<td>C. freundii</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. aspera</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

=> weights for each bin:
++ : highly species-specific (+15, +20, ...)
+ : moderately species-specific (+3, +4, ...)
- : peak absent (-3, -4, ...)
-- : Absent and peak important (-15)
Identifying the organisms

- Unknown microorganism is compared against reference library of spectra from culture collection strains
- ‘Goodness of fit’ is ranked and a threshold is applied for identification
- The majority of the peaks (~60-70%) are estimated to be ribosomal proteins, but for the most part, the protein for any individual peak is unknown

Impact of MALDI-TOF MS on blood culture TAT

- E. faecium: 22.2 h vs. 11.5 h
- C. parapsilosis: 12.9 h vs. 42.4 h
- E. coli: 14.3 h vs. 9.9 h
- E. cloacae complex: 15.1 h vs. 23.9 h
Impact of MALDI-TOF on urine culture TAT

Lessons from Microbiology: Widespread utilization of MALDI-TOF

People that love MALDI-TOF:
- Clinicians
- Medical Lab Directors
- Technical Directors
- Technologists/Microbiologists
- Residents/Fellows
Summary of Part 2: Current Clinical Applications

LC-MS/MS in clinical chemistry

MALDI-TOF MS in clinical microbiology

Why LC-MS/MS?
• More sensitive
• More specific
• More quantitative
• Multiplexed

Why MALDI-TOF?
• Faster
• More robust
• Answers the diagnostic question

Clinical Diagnostics

Pathology Department (BWH)

Anatomic Pathology (AP)

Clinical Pathology (CP) aka Lab Medicine
Diagnostic “Blind Spots”: Lipids

Revisiting Case 1

- Characteristic comprehensive features:
  - Clinical presentation
  - Radiographic features
  - Gross pathological features
  - Histopathological features

- By macromolecules
  - Immunohistochemistry: MIB-1, GFAP, OLIG2
  - Cytogenetic: 1p/19q deletions
  - Genetic: IDH mutational status
  - Epigenetic: MGMT promoter hypermethylation

Absent in this (and nearly all) pathology workups:
Lipid metabolism plays a critical role in numerous human diseases

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>Annual Mortality (in US)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart disease</td>
<td>597,689</td>
</tr>
<tr>
<td>Cancer</td>
<td>574,743</td>
</tr>
<tr>
<td>Chronic lower respiratory diseases</td>
<td>138,080</td>
</tr>
<tr>
<td>Stroke (cerebrovascular diseases)</td>
<td>129,476</td>
</tr>
<tr>
<td>Accidents (unintentional injuries)</td>
<td>120,859</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>83,494</td>
</tr>
<tr>
<td>Diabetes</td>
<td>69,071</td>
</tr>
<tr>
<td>Nephritis, nephrotic syndrome, and nephrosis</td>
<td>50,476</td>
</tr>
<tr>
<td>Influenza and Pneumonia</td>
<td>50,097</td>
</tr>
<tr>
<td>Intentional self-harm (suicide)</td>
<td>38,364</td>
</tr>
</tbody>
</table>

Lipids and cancer

- Not a new subject:

- Of course, a few years later, a slightly more prominent paper was published:

- ...and the era of genetics began...
Lipids largely left behind in cancer biology and diagnostics

Pubmed search (last night – 12.2.2020)

<table>
<thead>
<tr>
<th>Pubmed search terms</th>
<th>Unique publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>“cancer”</td>
<td>4,235,283</td>
</tr>
</tbody>
</table>

Why the lipidomics neglect? Reasons may be several-fold
**Complexity: Numerous metabolic pathways involved in lipid metabolism**

![Diagram showing metabolic pathways involving lipids](image)

- **Over 500 lipids measured in plasma**

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Formula</th>
<th>Molecular Weight</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>Choline-P C18:0</td>
<td>734.6</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>C27H44O3</td>
<td>428.65</td>
<td>Steroid lipid</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>C57H120O6</td>
<td>864.33</td>
<td>Triglyceride</td>
</tr>
</tbody>
</table>

On the other hand...

Historically, lipids have been difficult to measure

- Thin layer chromatography was mainstay of lipid analysis for many decades
The study of DNA and proteins found their “magic bullets”

Genomics:
DNA Polymerase/PCR

Proteomics:
Hybridoma/monoclonal Ab

Lipids found a more slowly developing partner in mass spectrometry

Several Nobel Prizes later

Mass spectrometer (circa 1940)

Mass spectrometer (present day)
Conventional methods to characterize lipids and metabolites in vitro

Issues with traditional LC-MS approaches to cancer lipidomics
Diagnostic "Blind Spots":

Cancer Metabolism/Metabolomics

Altered cellular metabolism is one of the oldest known hallmarks of cancer

1976: A diagnostic breakthrough made by visualizing cancer metabolism

- Pioneered by Dr. Abbas Alavi at Penn, Positron Emission Tomography (PET) was first used in humans in August 1976.

- Measuring FDG-glucose uptake allowed, for the first time, the detection of cancer using a metabolic footprint.

- Today, PET-CT has become one of the mainstays not only in cancer detection, but in monitoring of disease and assessment of therapy.

Unlike radiology, pathology lacks a method to assess cellular metabolism

- Some measures of cellular proliferation are utilized

- However, these are not indicative of cellular metabolism

- In essence, there is no "PET scan" in pathology
Major pathways to analyze

1. Glycolysis
2. PPP
3. Serine metabolism
4. Krebs Cycle
5. Glutaminolysis/RC
6. Fatty acid synthesis
7. BCAA metabolism
8. Aspartate metabolism

Current tools for the pathologist

Clinical Problem:
These methods provide limited information on cancer metabolism
Characterizing cancer metabolism

Targeting cancer metabolism is one of the great successes in cancer therapy

Aminopterin (modified to methotrexate)
Anabolic pathways

Proteins: 55%
Nucleic acids: 20%
Lipids: 10-20%


Diagnostic
"Blind Spots":

Rapid results
Revisiting Case 1

Diagnostics

- Molecular: MGMT, aCGH, next gen sequencing

Surgery for focal breast cancer

- Re-excision rates approach 20-40%*
- Can be reduced with additional cavity shave margins 34% → 19% (Chagpar et al NEJM, 2015)
- Novel techniques that can be used in the OR, such as mass spectrometry-based approaches, are continually sought


Re-excision rates approach 20-40%*

Can be reduced with additional cavity shave margins 34% → 19% (Chagpar et al NEJM, 2015)

Novel techniques that can be used in the OR, such as mass spectrometry-based approaches, are continually sought
Therapy is still driven by IHC and molecular classification

Receptor Status Molecular Classification

Rivenbark et al AJP (2013)

Diagnostic “Blind Spots”: Infectious Disease Pathology
Revisiting Clinical Case 2

- Delocalization
- Sensitivity
- TAT
- Expertise required
- Conidia needed
- Specificity
- Cost, labor, time
- Stability of nucleic acids during processing

Bacterial infections: Endocarditis
Acid fast organisms

Nocardia in the lungs

Mycobacteria in the liver

Parasites

Cryptosporidium in GI tract

Toxoplasma in the brain
Infectious workup from the OR

2 specimens (or 1 large specimen) → OR

Microbiology (CP)
- Grind up tissue
- Tissue fragment
- Micro specimen 1

Pathology (AP)
- Formalin Fixation, Paraffin embedding
- ‘Bug stains’
- ‘Permanent sections’
- Tissue Processing

Bacterial culture
- Frozen
- Micro Specimen 2

Fungal culture
- Gram
- Silver
- AFB

If (-) → Sent to specialty reference lab
If (+) → Sent to specialty reference lab

Microbes exhibit unique biochemistry

Viral

Mycobacterial

Bacterial

Fungal
Diagnostic “Blind Spots”:
Drug measurement in tissue

Revisiting case 3

Crystals
Another glioma case, part of a clinical trial

MRI- first surgery, treated
MRI- 8 month shows progression

- Is drug ineffective because of drug resistance?
- Is drug ineffective because of incomplete penetration?

Revisiting Clinical Case 2

Same questions:
- Is drug ineffective because of drug resistance?
- Is drug ineffective because of incomplete penetration?
## Coming up...

<table>
<thead>
<tr>
<th>Time (PST/PAC)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thursday 6:45 - 7:00 am</td>
<td>Pre-class networking and discussion</td>
</tr>
<tr>
<td>Thursday 7:00 - 8:00 am</td>
<td>Clinical tissue analysis: overview of workflow and diagnostic &quot;blind spots&quot; (MS)</td>
</tr>
<tr>
<td>Thursday 8:00 - 8:20 am</td>
<td>MS/MS</td>
</tr>
<tr>
<td>Thursday 8:20 - 9:00 am</td>
<td>Introduction to MALDI MS (MS)</td>
</tr>
<tr>
<td>Thursday 9:00 - 9:40 am</td>
<td>BREAK</td>
</tr>
<tr>
<td>Thursday 9:40 - 10:30 am</td>
<td>Adjacent and applications using MALDI MS (MS)</td>
</tr>
<tr>
<td>Thursday 10:30 - 11:00 am</td>
<td>MS/MS</td>
</tr>
<tr>
<td>Thursday 11:00 - 12:00 pm</td>
<td>Office hrs &amp; discussion</td>
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<tr>
<td>Friday 6:45 - 7:00 am</td>
<td>Pre-class networking and discussion</td>
</tr>
<tr>
<td>Friday 7:00 - 8:00 am</td>
<td>Introduction to ambient MS (EI)</td>
</tr>
<tr>
<td>Friday 8:00 - 8:20 am</td>
<td>BREAK</td>
</tr>
<tr>
<td>Friday 8:20 - 9:00 am</td>
<td>Advancements and applications using AMI (EI)</td>
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<tr>
<td>Friday 9:00 - 9:40 am</td>
<td>MS/MS</td>
</tr>
<tr>
<td>Friday 9:40 - 10:30 am</td>
<td>Bringing MS into the clinic: opportunities and challenges (MS)</td>
</tr>
<tr>
<td>Friday 10:30 - 11:00 am</td>
<td>MS/MS</td>
</tr>
<tr>
<td>Friday 11:00 - 12:00 pm</td>
<td>Office hrs &amp; discussion</td>
</tr>
</tbody>
</table>
Introduction to MALDI IMS

Michelle L. Reyzer, Vanderbilt University

Imaging techniques

*In vivo* (non-destructive)  
- PET  
- X-Ray  
- CT  
- MRI  
- Fluorescence  
- Ultrasound

*Ex vivo* (destructive)  
- Autoradiography

In most cases, these imaging techniques are non-specific  
- require a label (PET, MRI, fluorescence)  
- show structures without composition (ultrasound, x-ray/CT)
**PET and X-ray CT**

**Positron Emission Tomography**
- uses radioactive positron emitters
  - $^{15}$O – 2 min
  - $^{13}$N – 10 min
  - $^{11}$C – 20 min
  - $^{18}$F – 110 min
- $^{18}$F-FDG (fluorodeoxyglucose) commonly used to assess metabolic activity
- typically poor resolution (~1-4 mm)

**X-ray Computed Tomography**
- multiple X-ray images reconstructed in 3D
- better spatial resolution (~100 µm)
- only shows structure

Combined PET/CT scanners available to integrate the two technologies


**MRI**

**Magnetic Resonance Imaging**
- can image isotopes with nuclear spin
  - $^1$H
  - $^{13}$C
  - $^{19}$F
- contrast agents with paramagnetic atoms (Gd, Fe, Mn) are commonly used
- resolution ~0.7 – 2 mm
- shows structure of soft tissues
Autoradiography

- can image radioactive isotopes
  - $^3$H
  - $^{14}$C
  - $^{32}$P
- used ex vivo to monitor drug distribution in animals
- resolution $\sim 30 - 100 \ \mu$m
- only monitors the label

Rat 1 hr after iv administration of $^{14}$C-ethanol

Imaging with mass spectrometry

With so many other imaging techniques, why use MS?
- Combines molecular specificity with location
- Obtain a molecular picture
- A picture is worth a thousand words!

Differential expression of proteins along the length of the mouse epididymis.

Basic approach of Imaging MS

Obtain sample
• Tissue
• Plants
• Polymers
• Minerals

Prepare thin section for analysis

Desorb/ionize analytes
• Ion gun
• Laser
• Molecular beam

Acquire spectra over sample surface

Reconstruct two-dimensional ion images

A brief history of IMS

In the beginning there was... SIMS

• ~50 yrs ago (early 1960’s)
• Secondary ionic emission/ion microprobe mass analyzer
• Sputter ion source (duoplasmatron) – created a dense beam of gaseous ions (typically Ar⁺, O₂⁺, O⁻) as the primary bombarding ion beam
• Very energetic process – produces elemental ions and fragments
• Spatial resolution ~1-2 µm
• Microprobe or microscope mode

Microprobe

- Al⁺ ions in grid (gold between)
- 12 kV Ar⁺ primary in beam
- Beam focused to ~1.5 µm
- Vertical spacing 1.6 µm
- 300 x 300 µm total area
- 2 sec

Microscope

- Al⁺ ions underneath copper grid
- 10 kV Ar⁺ primary ion beam
- Spatial resolution better than 1 µm
- 5 sec

Al⁺ = m/z 27
Evolution of SIMS

1970's  Compared to established surface techniques
Advances targeted for resolution, sensitivity, and higher MW species
- $^{90}$Zr$^+$ and $^{56}$Fe$^+$ ions
- Andersen, CA and Hinthorne, JR, Science, 1972, 175(4024), 853

1980's  Higher spatial resolution
- Liquid metal ion guns
- Submicron imaging
- NbO$^+$ ions (m/z 109) in superconductor
- Ga$^+$ primary ion beam focused to 0.1 µm
- Waugh, AR et al, Vacuum, 1984, 34(1-2), 103

1990's  Subcellular imaging
- TOF analyzers
- A single MCF-7 mammary carcinoma cell dosed with 5-bromo-2'-deoxyuridine (BrdU) and 5-fluorouracil (FU)
- Cs$^+$ primary ion beam
- Image resolution better than 100 nm
- Berry, J-P et al, Biol Cell, 1992, 74, 93

2000's  Cluster ion sources
- Softer ionization
- Imaging with lasers
The first working laser was described in 1960
- LDI MS was described shortly thereafter (~1963-1964)
- Commercial LDI mass spectrometer available late 1970's (LAMMA)
- Spatial resolution ~1-5 µm
- Microprobe mode
- Somewhat less energetic than SIMS – still mostly small molecules
- Produces many neutrals – postionization strategies used
- Gentian violet dye
- [M+H$^+$ at m/z 372
- LAMMA-1000 (TOF)
- Nd:YAG (265 nm)
- Vertical spacing 25 µm
- 750 x 750 µm total area
- Wilk, ZA and Hercules, DM
  Anal Chem, 1987, 59(14), 1819

Apollo 11 moon rock

• $^{90}$Zr$^+$ and $^{56}$Fe$^+$ ions
• Andersen, CA and Hinthorne, JR, Science, 1972, 175(4024), 853
Laser ablation with positionization

**LA: CO₂ IR**
- Nd:YAG at 266 nm

**LA-ICP MS**
- Nd:YAG (1064 nm)
- ICP

**LAESI**
- CO₂ IR
- ESI

- Martian meteorite
- Polycyclic aromatic hydrocarbons (PAHs)
- Spacing 100 µm
- McKay, DS et al., Science, 1996, 273(5277), 524

- Salmon fish scale tagged with Sr
- 88Sr/48Ca
- Spacing 30 µm
- Wang, S et al., Appl Spectrosc, 1994, 48(11), 1321

- Plant leaf (zebra plant)
- Methoxykaempferol glucuronide (m/z 493)
- Spacing 400 µm
- 4 x 12 mm total area
- Nemec, P et al., Anal Chem, 2008, 80(12), 4575

**MALDI**

**Matrix-Assisted Laser Desorption/Ionization**
- Described in late 1980’s
- Employs a “matrix” – metal nanoparticles, organics, etc.
- Softer ionization than SIMS or LDI
- Allowed high MW compounds to be detected via LA

- Human cheek cells
- m/z 7,605
- Fragment of basic proline-rich peptide IB-1
- CHCA matrix
- N₂ laser
- TOF MS
- Caprioli, RM et al., Anal Chem, 1997, 69(23), 4751
Ambient ionization sources

DESI
- Rat brain
- Lipids
- Resolution better than 500 µm
- 13 mm x 10 mm area
- Wiseman, JM et al, Angew Chemie Int Ed 2006, 45, 7188

IR-LAMICI
- Tylenol tablet
- Acetaminophen
- Resolution ~300 µm
- 14 mm x 7 mm area

LMJ-SSP
- Ink on paper
- m/z 443 → 399

LTP
- Inkpad seal
- Ink components
- Resolution 150 µm
- Liu, Y et al, Angew Chemie Int Ed, 2010, 49, 4435

...and others!!

Impact of imaging MS

Increase in number of imaging publications

Number of publications

Impact of imaging MS

Increase in number of imaging publications

Number of publications

MSACL Connect: Imaging 101: Clinical Studies using Imaging MS ©2020 Sankha Basu, Michelle Reyzer, Christina Ferreira
MALDI
Matrix-Assisted Laser Desorption/Ionization

- Excellent for large molecules
- Soft ionization
- Moderate to high resolution (laser)
- Addition of matrix

Garrett, T. J. et al., Int. J. Mass Spectrom., 2007, 260, 166

Imaging in 4 (easy?) steps
1. Sample preparation
2. Matrix application
3. Instrument considerations
4. Data/image processing
1. Sample preparation

- The entire procedure from sample accrual, through sectioning, washing, and matrix application up until analysis.

**General goals**

1. Maintain molecular integrity (minimize degradation)
2. Maintain spatial integrity (minimize physical deformation)
3. Minimize analyte delocalization
4. Optimize sensitivity/resolution for desired experiment

Keep in mind...
- Some of these parameters may be beyond your control
- There are always trade-offs

---

**General procedure**

- Obtain tissue sample – freeze in liquid N₂
- Section frozen tissue on cryostat (~3-20 μm)
- Thaw-mount section onto target plate
- Pre-treat FFPE
- Apply matrix
- Acquire mass spectra
- Insert plate into mass spectrometer
- Data processing/analysis
Flash freezing and storage

- Immediately after dissection:
  - Wrap loosely in foil
  - Briefly expose tissue to dry ice vapor
  - Immerse in liquid nitrogen
  - Place on dry ice
  - Store at -80°C in “large” container
- Avoid small containers
- Always freeze first
- Small tissues may be frozen on a coverslip, then stored

Sample sectioning

- Mounted on cryostat block
- OCT used as “glue”
- 5-20 μm thick
- Artist’s paintbrush for manipulation

Frozen tissue (liver)

Multiple sections mounted on MALDI plate
Effect of OCT polymer on signal

- Tissue section did not come into contact with OCT
- Tissue section was embedded in OCT

Tissue mounting methods

- Some soluble proteins are lost with the "pick-up" method

* MSACL Connect: Imaging 101: Clinical Studies using Imaging MS ©2020 Sankha Basu, Michelle Reyzer, Christina Ferreira
A word about plates...

Plates must be compatible...
- with the instrument (size, conductivity)
- with the tissue (size)
- with the matrix application process

Plate possibilities:
- Instrument plates
- Inserts
- Plate adapters

Does conductivity matter?

Yes....
- Can lead to charging effects (peak broadening and mass shifting)
- Gets worse with higher frequency lasers
  \[\text{TOF, TOF/TOF}\]

average of 20 shots (3Hz)

Porcine insulin + SA
Non-conductive glass slide
Linear MALDI TOF MS
25 kV accelerating potential

...and No
- Some instruments do not apply extraction voltage to plate or use a lower voltage (typically those with decoupled source and mass analyzer)
  \[\text{ion trap, FT-ICR}\]
Sample pretreatment

Why pretreat?
- Remove interferences (salts, lipids, OCT)
  - reduce signal suppression
  - reduce adduct formation
  - improve on-tissue crystallization
  - better sensitivity
  - more signals
- Preserve sample for later analysis (dehydration)
- May be necessary for further processing (staining, LCM)

Why not?
- May induce delocalization
- Soluble analytes may be lost

Washing for membrane proteins

- Human lens, aquaporin 0 (AQP0)
- Tissues washed with water 10x (1 min each)
- Washing removes abundant, soluble proteins (crystallins)

Gray, A. C. et al, *J. Proteome Res.*, 2009, (8), 3278
Staining strategies

1. Is ROI big enough to appear on serial sections?
   - YES: Stain serial section
   - NO: Is staining required to visualize target?
     - YES: Use MALDI compatible stain prior to MS analysis
       - Minimize analyte loss
       - Minimize delocalization
       - Evaluate compatibility
     - NO: Stain tissue after MS analysis

2. Matrix application

- Matrix-assisted laser desorption/ionization

What is a matrix??
- Anything that assists with desorption and ionization!
- Generally thought to absorb light of the laser and gently transfer energy to analytes

Matrices perform additional functions in imaging...
- Extract analytes from tissue (solvent)
- Co-crystallize with analytes
Types of matrices

Traditional (UV)
- 2,5-dihydroxybenzoic acid (DHB)
- sinapinic acid (SA)
- α-cyano-4-hydroxycinnamic acid (CHCA)
- 2,4,6-trihydroxyacetophenone (THAP)
- 2,6-dihydroxyacetophenone (DHA)
- 3-hydroxypicolinic acid (HPA)
- ferulic acid (FA)

Traditional (IR)
- water
- succinic acid
- glycerol

Non-traditional (UV)
- metal particles (Ag, Co, Au, etc.)
- porphyrins
- solid supports (silicon - DIOS, NIMS)
- graphite/carbon nanostructures
- 9-aminoacridine (9AA)
- 1,5-diaminonaphthalene (DAN)

...and many more!

MALDI images

IR MALDI of strawberry skin
matrix: endogenous water

UV MALDI of Arabidopsis flowers
matrix: colloidal silver

UV MALDI of fingerprints
matrix: CHCA

UV MALDI of human invasive breast cancer
matrix: SA

Rauser, S. et al, J. Proteome Res., 2010, 9, 6317
Matrix application considerations

**Spotting**
- Apply discrete volumes of matrix solution to tissue
- Delocalization is limited to the area of the dried spot

**Coating**
- Apply a homogeneous coating of matrix to tissue
- Allows for higher spatial resolution imaging

**Dry**
- No solvent is used during matrix application
- No delocalization/no extraction

**Wet**
- Matrix is applied dissolved in solvent
- May induce delocalization/assist with analyte extraction

Matrix application considerations

Apply matrix

- Large droplet (nl)
  - Profiling
- Small droplet (pl)
  - Profiling/Imaging
- Uniform coating
  - Imaging

Acquire mass spectra and process data

- Protein profiles
- Images
Histology-Directed Protein Profiling

- One section on MALDI target
- Serial stained section
- Whole MALDI target
- Annotated histology section
- Merged pictures
- Robotic matrix spotting
- Spotting accuracy
- Mass spectral profiles

Matrix application: Robotic coating

- Commercial instruments available
  - Matrix is applied in cycles
  - Deposits pL to nL volumes
- Advantages
  - Automated
  - Reproducible process
- Disadvantages
  - Sample plate compatibility
  - One plate at a time
  - May give uneven coating
Matrix application: Sublimation

- Sublime matrix under vacuum/heat onto MALDI target
- No solvent!!
  - No delocalization
  - No real extraction*
- Excellent for lipids
- High-resolution imaging possible

*but can use recrystallization


Recrystallization

- Applied to pre- or post-coated tissues
- Analyte extraction occurs after matrix deposition
- Allows high resolution imaging

- Rat brain tissue
- SA
- Sublimation
- Recrystallized by exposing tissue to solvent vapor
  - 3-4 min
  - 85°C
  - 1 ml 5% acetic acid
  - 10 µm raster

A word about delocalization

General assumption: analytes delocalize within a spot

Matrix amount and tissue thickness

- Chicken liver tissue
- SA
- Sublimation
- Rehydration

Summary

♦ Keep in mind the overall goal of the experiment!
♦ The analyte of interest and desired spatial resolution will dictate the matrix, solvent, and application method

3. Instrument considerations

♦ Things to think about that affect the image set-up
MALDI for imaging

The basics:
• Requires a laser (UV most common)
• Requires a matrix (typically absorbs laser light)

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Characteristic</th>
<th>Impact on imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser</td>
<td>Frequency</td>
<td>Time</td>
</tr>
<tr>
<td></td>
<td>Wavelength</td>
<td>Matrix compatibility</td>
</tr>
<tr>
<td></td>
<td>Beam diameter</td>
<td>Resolution</td>
</tr>
<tr>
<td></td>
<td>Energy</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Matrix</td>
<td>Absorption profile</td>
<td>Laser compatibility</td>
</tr>
<tr>
<td>Solution</td>
<td>Analyte extraction (sensitivity)</td>
<td>Delocalization (resolution)</td>
</tr>
<tr>
<td>MW</td>
<td>Chemical interference (sensitivity)</td>
<td></td>
</tr>
</tbody>
</table>

MALDI for imaging

...but there’s more!

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Characteristic</th>
<th>Impact on imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass analyzer</td>
<td>Mass range</td>
<td>Analyte</td>
</tr>
<tr>
<td>Duty cycle</td>
<td>Data file format</td>
<td>Time</td>
</tr>
<tr>
<td>Data file format</td>
<td>File size, exportability</td>
<td>Resolution</td>
</tr>
<tr>
<td>x,y stage</td>
<td>Step size (precision)</td>
<td>Resolution</td>
</tr>
<tr>
<td>Dimensions</td>
<td>Software</td>
<td>Raster patterns</td>
</tr>
<tr>
<td>Software</td>
<td>Image area definition</td>
<td>Sensitivity/resolution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>File size, resolution</td>
</tr>
</tbody>
</table>
A few generalities...

In principle, IMS may be performed with any mass analyzer*

* certain terms and conditions may apply

<table>
<thead>
<tr>
<th>General</th>
<th>Source</th>
<th>Analyzer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automated data acquisition</td>
<td>Spatial resolution/focus</td>
<td>Mass resolution</td>
</tr>
<tr>
<td>Automated image processing</td>
<td>Ionization tendencies/analyte classes</td>
<td>MW range</td>
</tr>
<tr>
<td>Acquisition time</td>
<td>Acquisition time</td>
<td>Sensitivity</td>
</tr>
</tbody>
</table>

Image acquisition parameters

- Understand your experiment first!!
  - What question(s) are you trying to answer
  - How much resolution/sensitivity do you need?
- Laser spot size
- Laser repetition rate
- Spatial resolution
- Collection modes
- Analysis modes
- All of these will influence how the image is set up!
## Instruments used for IMS

<table>
<thead>
<tr>
<th>Mass Analyzer</th>
<th>Mass Resolving Power (m/Δm)</th>
<th>m/z range</th>
<th>Throughput (g/set)</th>
<th>Tandem MS Capabilities</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMS TOF</td>
<td>10^4</td>
<td>0 - 10^6</td>
<td>2 – 5</td>
<td>no</td>
<td>protein/protein digestion</td>
</tr>
<tr>
<td>HELION TOF</td>
<td>10^6 – 10^7</td>
<td>0 – 10^6</td>
<td>3 – 5</td>
<td>100 (protein digestion)</td>
<td>drug metabolites/proteins/lipids</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>10^9 – 10^10</td>
<td>100 – 10^6</td>
<td>&lt;1</td>
<td>100</td>
<td>drug metabolites/proteins/lipids</td>
</tr>
<tr>
<td>IMS TOF Plus</td>
<td>10^9 – 10^10</td>
<td>100 – 10^6</td>
<td>&lt;1</td>
<td>100</td>
<td>drug metabolites/proteins/lipids</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>10^9 – 10^10</td>
<td>100 – 10^6</td>
<td>&gt;100</td>
<td>100</td>
<td>drug metabolites/proteins/lipids</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>10^11 – 10^12</td>
<td>10^9 – 10^10</td>
<td>&lt;100</td>
<td>100</td>
<td>drug metabolites/proteins/lipids</td>
</tr>
</tbody>
</table>

Norris, J. L. and Caprioli, R. M.; Chem. Rev., 2013, 113 (4), 2309

## IMS Performance Criteria

### Traditional Considerations
- **Mass Resolving Power**: defined as $m/\Delta m$
- **Mass Accuracy**: the difference between the measured mass and the calculated exact mass
- **Sensitivity**: specifies the overall response of the instrument for a given analyte
- **Dynamic Range**: detection range for the instrument (most intense/smallest detectable signal)
- **MS^n capabilities**: ability to perform fragmentation experiments for analyte identification

### IMS Special Considerations
- **Spatial Resolution**: the distance between two adjacent pixels or ablated spots on the sample surface
- **Throughput**: the number of scans/spectra that can be acquired per unit time
- **File Size/Data Storage**: considerations of storage costs and processing practicality
Spatial resolution

Resolution may be described...

“mechanically”: by beam diameter and spacing

at the beam resolution

undersampling

oversampling

“optically”: by separation of definable features

Ultimately, resolution should be determined by what is needed to answer the analytical question
Imaging with high spatial resolution

Mouse Retina (10 µm)

CH = choroid
RPE = retinal pigment epithelium
OS/IS = photoreceptor outer and inner segments
ONL = outer nuclear layer

In this case, high spatial resolution is necessary to elucidate each retinal layer

Oversampling example

- 200 µm laser spot size
- 100 µm grid spacing
- Varied step size for oversampling
- Should completely ablate previous spot before stepping to next spot (lots of laser shots needed)

Other factors

- Sample preparation methods affect spatial resolution
  - Spray/sublimation coating (>1 µm)
  - Spotting (>250 µm)

- Mass spectrometer affects spatial resolution
  - Laser characteristics and optics (what is diameter of the laser on the target surface?)
  - Stage movements
  - Signal intensity

Acquisition Time vs. Lateral Resolution

- Biological Specimen
  - Scan Time (ST) = 1 s
  - 64 x 96 = 6144 pixels  
    102.4 min (total time)
  - 32 x 48 = 1536 pixels  
    25.6 min
  - 16 x 24 = 384 pixels  
    6.4 min
  - 8 x 12 = 96 pixels  
    1.6 min

- Density of pixels: pixels per inch (ppi)

\[
Time = ST \frac{X \cdot Y}{(\text{pixel size})^2}
\]
Spatial Resolution vs. Throughput

- ~345,000 pixels/3hr  
  Next Generation MALDI TOF
- ~21,000 pixels/3hr  
  MALDI TOF
- ~9,800 pixels/3hr  
  LIT
- ~7,700 pixels/3hr  
  FT-ICR
- ~7,200 pixels/3hr  
  IM Q-TOF

Main factors that determine data size:

1. extent of the scanned mass range
2. m/z resolution (m/z bin size)
3. extent of measured tissue surface area
4. spatial resolution (pixel size)
5. precision with which the ion counts are stored

Factors 1 and 2 influence the data size linearly.

Factors 3 and 4 influence the data size quadratically.

Factor 5 can usually not be influenced by the user.
Final thoughts on experimental set-up

- Design experiments based on the information that you want and the resolution you need.
- Not all experiments need to have 2 ppm mass accuracy and 5 μm spatial resolution!
- Do you need the same resolution across the whole tissue?
- Do you need to image the whole tissue?

4. Data/Image processing

Preprocessing

The final ion intensity value reported by the detector for a particular m/z is determined by many factors. Some are instrumental, some are related to the wet-lab preparation, others are biological.

The goal of preprocessing is to maximally remove undesirable (non-biological) variation or noise from the mass spectrum, in order to enable interpretation or statistical analysis of the biological variations.

Most preprocessing methods will attempt to counteract only a specific noise type, and as a result the preprocessing phase of a study can entail various steps.

Slides courtesy of: Raf Van de Plas, Delft University of Technology, Netherlands
Shannon Cornett, Bruker Daltonics
Preprocessing

- **baseline correction:** quantifying and removing the chemical/instrumental noise background;
- **normalization:** projecting peak heights from several spectra onto a common intensity scale;
- **alignment:** projecting several spectra onto a common m/z scale;
- **smoothing/de-noising:** removing ion detector and data acquisition induced jitter;
- **calibration:** adjusting the m/z values according to a set of known calibrants;
- **peak detection:** converting a mass spectral profile to a discrete set of peaks;

---

Baseline correction

**Baseline correction:** Quantifying and removing the chemical/instrumental noise background.

What does it do to the spectrum?

David Rizzo
Preprocessing

Normalization

**Normalization:**
Projecting peak heights from several spectra onto a common intensity scale.

What does it do to the spectra?
Scale according to e.g. Total Ion Current (TIC).

Alignment

**Alignment:**
Projecting several spectra onto a common m/z scale.

What does it do to the spectra?
Shift and warp m/z axis to remove small m/z shifts.
Preprocessing

Alignment example:
- origin: mouse brain
- spectrometer: MALDI-TOF
- matrix: DAN
- mass range: lipid range

2000 spectra/pixels from an imaging MS experiment

Preprocessing

Smoothing/de-noising

Smoothing:
- Removing ion detector and data acquisition induced jitter.

What does it do to the spectrum?

Fit a smooth curve to the spectrum (e.g. by LOWESS)

Caution: Smoothing can reveal the underlying peaks, but often also influences peak height! Its use needs to be evaluated case-to-case (e.g. qualitative vs. quantitative analysis).
Preprocessing

**Calibration**

*(Mass) Calibration:*
Adjusting the m/z values according to a set of known calibrant compounds.

What does it do to the spectrum?
Refit the m/z axis to put calibrant peaks at their known m/z value.

Preprocessing

**Peak Detection**

*Peak Detection:*
Converting a mass spectral profile to a discrete set of peaks.

What does it do to the spectrum?
Find the peaks in a spectral profile and return a list of peaks.
Preprocessing

Custom workflows

- R – http://www.r-project.org (free)
- MATLAB – http://www.mathworks.com (paid license)
  - Bioinformatics Toolbox
    - Baseline correction: `msbackadj()`
    - Normalization: `msnorm()`
    - Alignment: `msalign()`, `msalign()`
    - Smoothing: `mslowess()`, `mssgolay()`
    - Peak picking: `mspeaks()`

One way of getting IMS data into MATLAB: MSiReader by Robichaud et al., JASMS 2013.
One way of getting IMS data into R: Cardinal by Bemis et al., Bioinformatics 2015.

Imaging Mass Spectrometry Data

Data Formats

- BioMap/Analyze 7.5 format
  - Refurbished MRI format;
  - Can be exported to by several vendors;
  - Little meta-information;

- `imzML`
  - Complete description of IMS experiments;
  - In the process of adoption by various vendors;
  - Builds upon open HUPO standard formats;

Vendor-specific formats as well...

More information about formats and software: http://www.maldi-msi.org/
Imaging Mass Spectrometry Data

Navigation Software

- **Free**
  - **BioMap** (Novartis): http://www.maldi-msi.org
  - **Datacube Explorer** (AMOLF): http://www.maldi-msi.org
  - **MsiReader** (NC State University): http://www.msireader.com
  - **OpenMSI** (Lawrence Berkeley Nat. Lab): http://openmsi.nersc.gov
  - **Cardinal** (Purdue & Northeastern University): http://www.cardinalmsi.org
  - ...

- **Vendor-specific**
  - **HDImaging** (Waters)
  - **FlexImaging** (Bruker)
  - **ImageQuest** (Thermo)
  - **TissueView** (AB SCIEX)
  - ...

Advances and Applications in MALDI IMS

Michelle L. Reyzer, Vanderbilt University

Biological applications

- Host-pathogen interactions [proteins]
- Drug distribution – anti-retroviral [drugs]
- Lysosomal storage disease – [gangliosides]
- Malaria – liver stage [lipids]
Biological applications

Advances/Approaches

- Host-pathogen interactions [proteins] instrumentation
- Drug distribution – anti-retroviral [drugs] MS/MS
- Lysosomal storage disease – [gangliosides] sample prep
- Malaria – liver stage [lipids] experimental design

Application: Host-Pathogen Interactions

- Methicillin-resistant Staphylococcus aureus (MRSA) is classified as a serious threat to public health by the CDC
Application: Host-Pathogen Interactions

- Using high mass-resolving power FT-ICR for imaging furthered our understanding of abscess organization

• S100A8 is recruited to abscesses in a neutrophil-dependent manner

Corbin, BD et al, Science, 2008, 319, 962

Spraggins, JS et al, JAMS, 2015, 26, 974
### Application: Host-Pathogen Interactions

**Proteins identified by accurate mass and correlated with parallel top-down proteomics experiments**

**High mass resolving power enabled unique protein species to be visualized**

**The advanced oxidation product of S100A8 (M37O/C42O3) is located specifically to the center of infectious foci**

Spraggins, JS et al, JASMS, 2015, 26, 974

### Application: Host-Pathogen Interactions

**Multi-Modal 3D Kidney Imaging**

- Acquired data for 48 kidney sections at 50 µm spatial resolution
- Total of 930,398 pixels
- Using continuous raster scanning (TOF)

Cassat, JE et al, Sci Transl Med, 2018, 10, eaan6361
**Application: Host-Pathogen Interactions**

- **IMS/optical blockface**
  - m/z 10,164 (S100A8)
  - Co-localizes with abscesses

- **IMS/MRI**

Proteins were found localized to the staphylococcal microcolony in the center of the abscesses and later identified as bacterial proteins

- m/z 6,888 – identified as CsbD-like protein; bacterial stress response
- m/z 3,006 – identified as δ-hemolysin; virulence factor

*Cassat, JE et al, Sci Transl Med, 2018, 10, eaan6361*
Application: Host-Pathogen Interactions

HOST: m/z 10,064 (blue)
PATHOGEN: m/z 3,006 (pink)

Application: Host-Pathogen Interactions

FT-ICR MS imaging of one whole-body section

Cassat, JE et al, Sci Transl Med, 2018, 10, eaan6361
Application: Host-Pathogen Interactions

Conclusions

• IMS has enabled the visualization of the host-pathogen response \textit{in vivo}
• Host immune response proteins have been shown accumulating at the site of infection
• Bacterial proteins have been shown localized to the infectious foci
• Coupling these results with other imaging modalities has enabled a deeper understanding of the infection process
• Ultimately, this may be helpful in designing new anti-microbial compounds

Application: Drug Distribution

Global HIV/AIDS epidemic

• An estimated 1.8 million individuals worldwide became newly infected with HIV in 2016
• This includes 160,000 children, most of whom were infected by their HIV-positive mothers

HIV PrEP (Pre-exposure prophylaxis)

• Truvada is a daily pill used for PrEP – FDA approved in 2012
• Tenofovir disoproxil fumarate (TDF) + emtricitabine (FTC)
• Must be taken every day – doesn’t always happen

Application: Drug Distribution

HIV PrEP

Sustained release products

• Integrated Pre-Clinical/Clinical Program for HIV Microbicides and Biomedical Prevention (IPCP-MBP, U19)
• Multi-institutional program led by Oak Crest Institute of Science in Monrovia, CA
• Goal to create a sustained drug delivery system – intravaginal ring (IVR) – to provide protection from HIV infection for an extended period of time (30 day)

PI: Marc Baum, Oak Crest Institute of Science

Pod-IVR Components

• Polymer-coated drug cores, referred to as “pods”
• An un-medicated, torus-shaped elastomeric support to hold the pods
• One or more delivery channels through the elastomer ring structure to provide the primary release rate control and link the pods to vaginal fluids

Tenofovir disoproxil fumarate and metabolites

MALDI imaging of low molecular weight compounds

- Spectral interferences from the matrix cause a loss of sensitivity and specificity
Approaches to minimizing matrix interferences

- Careful choice of matrix (pick a blank area)
- Use high resolution/high mass accuracy instrument (FT-ICR)
- Matrix-free techniques (LDI, DIOS)
- Non-traditional matrices (high MW porphyrins, carbon/graphite)
- Matrix additives (matrix suppression)
- MS/MS (pseudo-selected reaction monitoring)

MS/MS method development for targeted drug imaging

**TENOFOVIR**

**C<sub>7</sub>H<sub>12</sub>N<sub>3</sub>O<sub>3</sub>P**

MW<sub>im</sub> = 287.078

**NEGATIVE mode:**

m/z 286 →

**POSITIVE mode:**

m/z 288 →
Experimental design

Biological experiment
- Pod-IVR with TDF + lactate
- Sheep study
- Removed on day 28 (no washout)
- Terminal study – entire vaginal tract removed and fresh frozen

IMS experiment
- Tissue sectioned at 12 µm on a cryostat
- 9AA matrix applied via TM Sprayer (5 mg/ml, 90% methanol, 4 passes)
- MALDI LTQ XL (Thermo)
- Negative ion mode
- MS/MS

Biological experiment

<table>
<thead>
<tr>
<th>Sheep Study IVR Formulation</th>
<th>TDF</th>
<th>lactic acid</th>
<th>MCC</th>
<th>Mg stearate</th>
<th>pod coating</th>
<th>in vitro release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75%</td>
<td>18.5%</td>
<td>6%</td>
<td>0.5%</td>
<td>polyvinyl alcohol (PVA)</td>
<td>4.61 mg/d</td>
</tr>
</tbody>
</table>

TDF-lactic acid pod-IVR configuration
- 4 TDF-LA pods per IVR (~135 mg TDF, 33 mg L-lactic acid)
- three delivery channels per pod (1.5 mm diameter)

28 Day sheep study design

* Whole vaginal, rectal tracts collected at necropsy (1/3 animals – R63)
Sheep vaginal tissue

TFV distribution
\[ m/z \ 286 \rightarrow m/z \ 134 \ + \ m/z \ 151 \]

Intensity scale: 100-1e3

1 mm 100 µm
TDF: not detected

m/z 520 →

optical | H&E | m/z 270 | m/z 288 | m/z 404

Dosed

Control

Intensity scale: 0-4.35e2 0-2.277e2 0-3.94e2 0-4.535e2

100 µm

TDF-monoPOC: not detected

m/z 402 →

optical | H&E | m/z 372 | m/z 358 | m/z 328 | m/z 268

Dosed

Control

Intensity scale: 0-3.921e2 0-2.251e3 0-7.884e2 0-2.575e2

100 µm
TVFp distribution
m/z 366 → m/z 348

TVFpp distribution
m/z 446 → m/z 159 + m/z 348
Pharmacokinetics

\[ \text{In vivo TDF release rate} \]

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>2.0</td>
</tr>
<tr>
<td>P57</td>
<td>1.5</td>
</tr>
<tr>
<td>R63</td>
<td>1.0</td>
</tr>
</tbody>
</table>

- determined from measurement of residual TDF in IVRs following Day 28 removal

\[ \text{Vaginal tissue biopsy} \]

\[ \text{[analyte] (ng mg}^{-1} \text{ or fmol mg}^{-1}) !\]

\[ \text{Time (d)} \]

- TFV
- TFV-DP

Conclusions

- Successfully imaged TFV, TFVp, and TFVpp in sheep vaginal tissue dosed with pod-IVR
- Images show clear localization to the epithelium/mucosa
- In good general agreement with quantitative HPLC-MS results
- Images reveal some heterogeneity

Next up → human 28-day clinical trial with TDF-FTC ring (biopsies)
Application: Lysosomal Storage Disease
Mucopolysaccharidosis type II (MPS II)

- LSDs: over 50 monogenic disorders, most of which result from a deficiency of a single lysosomal enzyme
- Effects of enzyme inactivity result in accumulation of substrates, which often results in progressive central nervous system dysfunction
- MPS II is caused by a deficiency of iduronate 2-sulfatase (IDS), an enzyme involved in the catabolism of glycosaminoglycans (GAGs)
- MPS II occurs in an estimated 1 in 170,000 live births; ~2/3 of patients display the neuronopathic form with progressive cognitive impairment, behavioral symptoms, and death typically occurring in the second decade of life


Application: Lysosomal Storage Disease
Mucopolysaccharidosis type II (MPS II)

- Lysosomal malfunction also causes accumulation of secondary lysosomal storage products, including gangliosides
- Current standard of care is delivery of recombinant IDS, which successfully reduces GAG accumulation in peripheral tissues, but is ineffective in treating CNS components of the disease
- Denali Therapeutics has developed a novel enzyme transport vehicle (ETV) for CNS delivery of protein therapeutics
- Their novel compound (ETV:IDS) was evaluated in a mouse model of MPS II

Application: Lysosomal Storage Disease

- Mouse model

- GAGs are increased in most tissues in the IDS KO mouse
- Treatment of IDS KO mice with ETV:EDS significantly lowers brain GAGs, compared to treatment with IDS


Application: Lysosomal Storage Disease

- HPLC-MS/MS analysis reveals an increase in two isoforms of GM3 in brain
- The novel compound ETV:IDS reduces the level to normal

Application: Lysosomal Storage Disease

- Where are the GMs increased in the brain? Is the decrease observed with ETV:IDS localized? USE IMS!
- The problem: Gangliosides are often suppressed by abundant phospholipids!

Variables tested (ammonium formate wash):
1. washing time (0-90 sec)
2. concentration (1 – 100 mM)
3. wait time after matrix deposition until analysis (0, 24 hr, 48 hr at -20°C)


Yang, E et al, Int J Mass Spectrom, 2019,
Application: Lysosomal Storage Disease

- Imaging reveals KO mice have elevated GMs in the hypothalamus and amygdala brain regions
- Four weekly doses of ETV:IDS qualitatively reduced the accumulation of these ganglioside species to amounts seen in control mice; while only modest reductions were observed for IDS
Application: Lysosomal Storage Disease

Conclusions

- IMS has enabled the visualization of secondary lysosomal storage substrates in vivo in a mouse model of MPS II
- IMS supported the HPLC-MS/MS analysis of brain homogenates
- This information may be helpful in the drug discovery process

Application: Malaria host-parasite interaction

- In 2017, there were an estimated 219 million cases of malaria and 435,000 deaths
- Most deaths were children under age 5
- Caused by parasitic protozoa (Plasmodium)
- The liver stage of the life cycle is a logical point of intervention

PI: Alexis Kaushansky, Center for Infectious Disease Research
Application: Malaria host-parasite interaction

Image analysis of infected mouse liver tissue

- Imaging whole tissue sections is not efficient!

Application: Malaria host-parasite interaction

Approach: Targeted imaging analysis utilizing autofluorescence

- Representation of targeted foci in a single tissue section.
- Red ROIs indicate positive mode analysis
- Blue ROIs indicate negative mode analysis
- Several serial sections were analyzed per ITO
- 4 ITOs per acquisition

<table>
<thead>
<tr>
<th>Subsection Imaging TOF</th>
<th>Targeted Analysis FT-ICR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Plasmodia detected</td>
<td>21 Plasmodia detected</td>
</tr>
<tr>
<td>12,045 pixels analyzed</td>
<td>1,628 pixels analyzed</td>
</tr>
<tr>
<td>~30 minute acquisition time</td>
<td>~12 minute acquisition time</td>
</tr>
</tbody>
</table>
Application: Malaria host-parasite interaction

Autofluorescence

- Advantages
  - no additional sample prep
  - can be done on the same section that is imaged
  - provides rich histology
- Disadvantages
  - may not show features of interest
  - takes time (depending on scanner)

Notes
- Infected hepatocytes are small ~10-60 µm
- Infected hepatocytes do not always show up on AF

Patterson, N.H. et al., Anal. Chem., 2018, 90, 12395
Patterson, N.H. et al., Anal. Chem., 2018, 90, 12404
Application: Malaria host-parasite interaction

Conclusions

- A targeted histology-directed workflow utilizing autofluorescence enables an efficient method to analyze plasmodium infected hepatocytes
- The molecular characterization of infected hepatocytes can be assessed *in vivo*
Conclusions

• Measures native molecular distributions, providing new biological insights that easily correlate with other imaging modalities

• Is an excellent discovery technology because no target-specific reagents are needed

• Has exceptionally high throughput (in some cases less than a few seconds for data acquisition per sample), providing multiple images simultaneously at discrete MW values

• Can undergo fusion with data from other imaging modalities to create new imaging paradigms

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Ambient Ionization in the MS Analysis Workflow

**NO SAMPLE PREPARATION**
- Liquid Chromatography
- Gas Chromatography

**Sample introduction**
- Matrix-assisted desorption/ionization (MALDI)
- Electrospray (ESI)

**Ion source**
- AMBIENT IONIZATION METHODS

**Mass analyzer**
- Time of Flight (TOF)
- Quadrupole
- Quadrapole ion trap
- Triple Quadrupole
- Linear and 3D Ion Trap
- ICR
- Obritrap

**Detector**
- Secondary electron multiplier
- Faraday cup
- Scintillator
- Micro channel plate

**Vacuum system**
- Turbo pumps
- Rough pumps

**Data processing**

**GENERAL PRINCIPLE OF AMBIENT IONIZATION IS TO PRODUCE GAS-PHASE IONS FROM RAW, UNTREATED SAMPLES**
What Does Ambient Ionization Mean?

SAMPLE PROCESSING TAKES PLACE IN REAL TIME AND PROXIMAL TO IONIZATION, I.E. SAMPLE PROCESSING TAKES PLACE DURING THE ANALYSIS.

• Sample dilution, preconcentration, liquid-solid extraction: Desorption electrospray ionization (DESI), nano-DESI.
• Liquid-liquid extraction: liquid-DESI, extractive electrospray ionization (EESI).
• Filtration: Paper spray (PS).
• Thermal Desorption: Direct analysis in real time (DART), Atmospheric solid analysis probe (ASAP).
• Spallation by energy sudden desorption: Laser ablation electrospray ionization (LAESI), matrix-assisted laser desorption electrospray ionization (MALDESI), laser ablation coupled to a flowing atmospheric pressure afterglow (LA-FAPA).

Prof. R.G. Cooks index card (1981)

All ambient analysis methods produce ions at atmospheric pressure, or in the intermediate pressure region of the ion inlet during sample and ion transport into the mass spectrometer.

Ions can be produced by ion-molecule reactions, photochemical ionization, charged droplets by electrospray or sonic spray ionization mechanisms.

Ambient ionization methods differ in the real-time sample processing and ionization mechanisms

- There are over 50 ambient ionization methods: First ones were Desorption electrospray ionization (DESI) was introduced in 2004 and Direct Analysis in Real Time (DART) in 2005.
- Minimal amounts of solvent (microliter-level), short (1 sec to 2 min) analysis time.
- Suitable for combination with portable mass spectrometers.
- Spray-based methods: highly compatible with clinical studies due to applicability to raw biofluid and tissue analysis. Can be used for imaging or profiling analysis.

Nano-DESI: Variant of DESI that uses liquid microjunction to facilitated analyte desorption/extraction transfer and ionization.

ESI-like mechanisms (black)
APCI-like mechanism (blue)

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Extended name</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DESI</td>
<td>Desorption electrospray ionization</td>
<td>2004</td>
<td>(1)</td>
</tr>
<tr>
<td>DeSSI</td>
<td>Desorption sonic spray ionization</td>
<td>2006</td>
<td>(2)</td>
</tr>
<tr>
<td>DAPI</td>
<td>Desorption atmospheric pressure photodissociation</td>
<td>2007</td>
<td>(3)</td>
</tr>
<tr>
<td>EASI</td>
<td>Easy ambient sonic-spray ionization</td>
<td>2008</td>
<td>(4)</td>
</tr>
<tr>
<td>JedLI</td>
<td>Jet desorption electrospray ionization</td>
<td>2008</td>
<td>(5)</td>
</tr>
<tr>
<td>TM-DESI</td>
<td>Transmission mode desorption electrospray ionization</td>
<td>2008</td>
<td>(6)</td>
</tr>
<tr>
<td>LMJ-SSP</td>
<td>Liquid microjunction-surface sampling probe</td>
<td>2009</td>
<td>(7)</td>
</tr>
<tr>
<td>DICE</td>
<td>Desorption ionization by charge exchange</td>
<td>2010</td>
<td>(8)</td>
</tr>
<tr>
<td>Nano-DESI</td>
<td>Nanospray desorption electrospray ionization</td>
<td>2010</td>
<td>(9)</td>
</tr>
<tr>
<td>EADESI</td>
<td>Electrode-assisted desorption electrospray ionization</td>
<td>2010</td>
<td>(10)</td>
</tr>
<tr>
<td>APTDCI</td>
<td>Atmospheric pressure thermal desorption chemical ionization</td>
<td>2011</td>
<td>(11)</td>
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<tr>
<td>V-EASI</td>
<td>Vector easy ambient sonic-spray ionization</td>
<td>2011</td>
<td>(12)</td>
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<tr>
<td>APA</td>
<td>Air flow-assisted ionization</td>
<td>2011</td>
<td>(13)</td>
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<tr>
<td>LESA</td>
<td>Liquid extraction surface analysis</td>
<td>2011</td>
<td>(14)</td>
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<tr>
<td>PFC-ESI</td>
<td>Powder flow column electrospray ionization</td>
<td>2012</td>
<td>(15)</td>
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<td>AFADESI</td>
<td>Air flow-assisted desorption electrospray ionization</td>
<td>2013</td>
<td>(16)</td>
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<tr>
<td>DSSVI</td>
<td>Desorption electro-flow focusing ionization</td>
<td>2014</td>
<td>(17)</td>
</tr>
<tr>
<td>LSITDI</td>
<td>Elaborates spray ionization transmission</td>
<td>2014</td>
<td>(18)</td>
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**Acronym, Names, Year of First Reporting, and Main References**

**Laser**

<table>
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<th>Acronym</th>
<th>Extended name</th>
<th>Year</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>MALDESI</td>
<td>Matrix assisted laser desorption electrospray ionization</td>
<td>2006</td>
<td>(34)</td>
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<tr>
<td>ELDESI</td>
<td>Electrospray laser desorption ionization</td>
<td>2007</td>
<td>(35)</td>
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<tr>
<td>LDSTD</td>
<td>Laser diode thermal desorption</td>
<td>2007</td>
<td>(36)</td>
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<tr>
<td>LAAESI</td>
<td>Laser ablation electrospray ionization</td>
<td>2007</td>
<td>(37)</td>
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<tr>
<td>CALDI</td>
<td>Charge assisted laser desorption ionization</td>
<td>2008</td>
<td>(38)</td>
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<tr>
<td>LA-FAPA</td>
<td>Laser ablation atmospheric pressure afterglow</td>
<td>2008</td>
<td>(39)</td>
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<td>LADESI</td>
<td>Laser assisted desorption electrospray ionization</td>
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<td>LDDESI</td>
<td>Laser desorption electrospray ionization</td>
<td>2009</td>
<td>(41)</td>
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<td>LEIMS</td>
<td>Laser electrospray mass spectrometry</td>
<td>2009</td>
<td>(42)</td>
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<td>LSI</td>
<td>Laser spray ionization</td>
<td>2009</td>
<td>(43)</td>
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<tr>
<td>IR-LAMIC</td>
<td>Infrared laser ablation metastable induced chemical ionization</td>
<td>2010</td>
<td>(44)</td>
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<tr>
<td>LDSPI</td>
<td>Laser desorption spray post-ionization</td>
<td>2010</td>
<td>(45)</td>
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<td>PAMLDI</td>
<td>Plasma assisted multiwavelength laser desorption ionization</td>
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<td>(46)</td>
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<td>HALDI</td>
<td>High voltage-assisted laser desorption ionization</td>
<td>2013</td>
<td>(47)</td>
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<tr>
<td>PALDI</td>
<td>Plasma assisted laser desorption ionization</td>
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**Spray-based**

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<tr>
<td>ESSI</td>
<td>Extractive electrospray ionization</td>
<td>2006</td>
<td>(49)</td>
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<td>PESI</td>
<td>Probe electrospray ionization</td>
<td>2007</td>
<td>(50)</td>
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<tr>
<td>ND-ESSI</td>
<td>Neutral desorption extractive electrospray ionization</td>
<td>2007</td>
<td>(51)</td>
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<tr>
<td>PS</td>
<td>Paper spray</td>
<td>2010</td>
<td>(52)</td>
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<tr>
<td>DIP-APCI</td>
<td>Direct inlet probe-atmospheric pressure chemical ionization</td>
<td>2013</td>
<td>(53)</td>
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<td>TS</td>
<td>Touch spray</td>
<td>2014</td>
<td>(54)</td>
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<tr>
<td>Wooden-tip</td>
<td>Wooden-tip electrospray</td>
<td>2014</td>
<td>(55)</td>
</tr>
</tbody>
</table>

Ferreira et al., Clinical Chemistry, Volume 62, Issue 1, 1 January 2016, Pages 99–110
## Acronyms, Names, Year of First Reporting, and Main References

<table>
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<th>Main Reference</th>
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<tbody>
<tr>
<td>RADIO</td>
<td>Radiofrequency acoustic desorption ionization</td>
<td>2009</td>
<td>(58)</td>
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<tr>
<td>LIAD-ESI</td>
<td>Laser induced acoustic desorption electrospray ionization</td>
<td>2009</td>
<td>(59)</td>
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<tr>
<td>SAWN</td>
<td>Surface acoustic wave nebulization</td>
<td>2010</td>
<td>(60)</td>
</tr>
<tr>
<td>UASI</td>
<td>Ultrasound-assisted spray ionization</td>
<td>2010</td>
<td>(61)</td>
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<tr>
<td>SPA-nanoESI</td>
<td>Solid probe assisted nanoelectrospray ionization</td>
<td>2012</td>
<td>(62)</td>
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<tr>
<td>PAUSI</td>
<td>Paper assisted ultrasonic spray ionization</td>
<td>2014</td>
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</table>

## Some Clinically Relevant Ambient Ionization Techniques and Applications

<table>
<thead>
<tr>
<th>Ambient ion source</th>
<th>Year</th>
<th>Compounds</th>
<th>Biological matrix</th>
<th>Sample volume</th>
<th>LOD</th>
<th>MS data acquisition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DART</td>
<td>2005</td>
<td>Ranitidine (exo)</td>
<td>Urine</td>
<td>—</td>
<td>Qualitative</td>
<td>LIT (exact mass measurement)</td>
<td>Gómez-Ríos et al. (30)</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>Propranolol, atenolol (exo)</td>
<td>DBS</td>
<td>—</td>
<td>Qualitative</td>
<td>QQQ-MS (MRM)</td>
<td>Wanger et al. (20)</td>
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<tr>
<td></td>
<td>2008</td>
<td>DOPA, ephedrine, and ibuprofen</td>
<td>DBS</td>
<td>—</td>
<td>Qualitative</td>
<td>Ranc et al. (13)</td>
<td></td>
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<tr>
<td></td>
<td>2014</td>
<td>Cocaine, diazepam (exo)</td>
<td>Urine, plasma</td>
<td>0.3–1.5 mL</td>
<td>Quantitative</td>
<td>MRM</td>
<td>Gómez-Ríos et al. (71)</td>
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<tr>
<td></td>
<td>2013</td>
<td>Phenylalanine PKU screening (endo)</td>
<td>DBS</td>
<td>—</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Gómez-Ríos et al. (71)</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Nicotine, cotinine, trans-3′-hydroxycotinine, anabasine (exo)</td>
<td>Blood, oral fluid, urine</td>
<td>5 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Wang et al. (74)</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Acylcarnitines (endo)</td>
<td>Blood, serum</td>
<td>0.5 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Esay et al. (29)</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Methadone, amitriptyline, nortriptyline, and pethidine</td>
<td>Urine</td>
<td>150 μL</td>
<td>Qualitative</td>
<td>LIT (full scan)</td>
<td>Rosting et al. (30)</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>Sunitinib and benzethonium (exo)</td>
<td>Plasma</td>
<td>2 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Takyi-Williams et al. (76)</td>
</tr>
<tr>
<td></td>
<td>2007</td>
<td>Androstadienedione, stigmastadienone, androsterone hemisuccinate, 5α-androstan-3β,17β-diol-16-one, androsterone glucuronide, epitestosterone, and 6-β-testosterone</td>
<td>Urine, serum</td>
<td>10 μL</td>
<td>Qualitative</td>
<td>QQQ-MS (MRM)</td>
<td>Wang et al. (74)</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Dimethylamylamine (exo)</td>
<td>Urine</td>
<td>—</td>
<td>Qualitative</td>
<td>HR TOF-MS (exact mass measurement)</td>
<td>Loiseau et al. (20)</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>Tacrolimus (exo)</td>
<td>DBS</td>
<td>10 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Edelbroek et al. (33)</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Phenylalanine PKU screening (exo)</td>
<td>DBS</td>
<td>—</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Wang et al. (74)</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>Nicotine, cotinine, trans-3′-hydroxycotinine, anabasine (exo)</td>
<td>Blood, oral fluid, urine</td>
<td>5 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Wang et al. (74)</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>Acylcarnitines (C2–C18)</td>
<td>Urine</td>
<td>Quantitative</td>
<td>6–208 ng/mL</td>
<td>QQQ-MS (MRM)</td>
<td>Naccarato et al. (75)</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>Lipid profile of pathogenic microorganisms</td>
<td>Oral fluid</td>
<td>40 μL</td>
<td>Qualitative</td>
<td>LIT (full scan)</td>
<td>Jarmusch et al. (26)</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Acylcarnitines (endo)</td>
<td>Blood, oral fluid, urine</td>
<td>0.5 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Espy et al. (29)</td>
</tr>
<tr>
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<td>2011</td>
<td>Acetaminophen, bezethonium, citalopram, dextrorphan, ibuprofen, paclitaxel, irinotecan, docetaxel, proguanil, simvastatin, sunitinib, telmisartan, verapamil, sitamaquine, amitriptyline (exo)</td>
<td>Urine</td>
<td>10 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Lesiak et al. (70)</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>Nicotine, cotinine, trans-3′-hydroxycotinine, anabasine (exo)</td>
<td>Blood, oral fluid, urine</td>
<td>5 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Wang et al. (74)</td>
</tr>
<tr>
<td></td>
<td>2007</td>
<td>Androstadienedione, stigmastadienone, androsterone hemisuccinate, 5α-androstan-3β,17β-diol-16-one, androsterone glucuronide, epitestosterone, and 6-β-testosterone</td>
<td>Urine, plasma</td>
<td>100 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Zhang et al. (36)</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>Sunitinib and benzethonium (exo)</td>
<td>Plasma</td>
<td>2 μL</td>
<td>Quantitative</td>
<td>QQQ-MS (MRM)</td>
<td>Takyi-Williams et al. (76)</td>
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<tr>
<td></td>
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<td>2014</td>
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</tr>
<tr>
<td></td>
<td>2013</td>
<td>Acylcarnitines (C2–C18)</td>
<td>Urine</td>
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<td>6–208 ng/mL</td>
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<tr>
<td></td>
<td>2014</td>
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<td>Oral fluid</td>
<td>40 μL</td>
<td>Qualitative</td>
<td>LIT (full scan)</td>
<td>Jarmusch et al. (26)</td>
</tr>
</tbody>
</table>

**Ferreira et al., Clinical Chemistry, Volume 62, Issue 1, 1 January 2016, Pages 99–110**
Advantages of Ambient MS methods

- **Simpler workflow, easier operation, does not need extensive training:** Seek to minimize or eliminate sample processing and incorporated it into the analysis.

- **Compatibility with MS imaging:** Processing steps are combined and localized and many of the ambient MS methods.

- **Suitability for high-throughput analysis:** Generate low sample carryover and interface contamination because samples stay out of the system until their concurrent processing and ionization occurs.

Ferreira et al., Analytical Letters, 47:1, 91-101

Overlay of DESI-MS ion images in the negative and positive ion modes as well as ion and optical images obtained from circa 3 mm² areas to observe particulate composition of the tablet’s ingredients.

Ambient Ionization and Miniature/Portable Mass Spectrometers

**MS-based profiling and quantification of molecules:** Usually by LC-MS/MS, provide selectivity, accuracy, precision and multiplexing.

Complexity of instrumentation and operation are challenging for POC development.

Li et al., Anal. Chem. 2014, 86, 6, 2909–2916
Ambient Ionization for Point-of-Care (POC) Diagnosis

POC testing: mostly assays for blood or urine using immunoassays or colorimetric screening.

- Doctor’s office
- Laboratory
- Operating room

Ambient Ionization: Compared to LC-MS/MS, the analysis workflow is more compatible with POC needs.

Schematic of sampling from culture or patient throat swab and subsequent analysis by touch-spray (TS)-MS

Negative ion mode mass spectrum of a single colony of S. pyogenes sampled from culture.

Jarmusch et al. Analyst 2014;139:4785–9

On-Line Derivatization Reactions for Biomolecules by ambient MS

Conventional DESI (ACN/H₂O/DMF)

Reactive DESI (ACN/H₂O/DMF with 50 ppm BA)

### Examples of On-Line Reactions for Biomolecules by Ambient MS

<table>
<thead>
<tr>
<th>Functional Groups</th>
<th>Reagents</th>
<th>Target compounds</th>
<th>Ambient MS method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldehydes and ketones</td>
<td>Girard’s reagent T, hydroxylamine, dinitrophenylhydrazine</td>
<td>Cortisone in oral fluid, steroid hormones [Huang et al. (72)], malondialdehyde in tissue [Girod et al. (77)]</td>
<td>PS, DESI</td>
</tr>
<tr>
<td>Alcohols</td>
<td>Betaine aldehyde</td>
<td>Cholesterol in tissue [Wu et al. (45)]</td>
<td>DESI</td>
</tr>
<tr>
<td>Amines</td>
<td>Bis(sulfosuccinimidyl) suberate, acetone</td>
<td>Cross-linking of peptides containing asparagine, glutamine, arginine, or lysine, amino acids [Gómez-Ríosa et al. (37)]</td>
<td>DESI</td>
</tr>
<tr>
<td>Disulfides and thiols</td>
<td>Dithiothreitol</td>
<td>Oxidized glutathione and insulin [Peng et al. (43)]</td>
<td>ELDI</td>
</tr>
<tr>
<td>Alkenes</td>
<td>Ozone, silver nitrate</td>
<td>Unsaturated lipids in bacteria, algae and animal preimplantation embryos [González-Serrano et al. (40), Zhang et al. (41), Jackson et al. (42)]</td>
<td>LTP, PS, DESI</td>
</tr>
</tbody>
</table>

**Ambient ionization by Liquid Extraction with Substrate Spray**

Specially suited for POC: No need of nebulizing gases

Paper Spray: Paper triangle can be sample storage. It is the emitter and promotes some sample separation.

---

Ambient ionization by Liquid Extraction and Desorption

- **Spray-Based Techniques**
  - DESI: 150-250 μm / 35 μm
  - EASI: ~200 μm
  - AFADESI: 300 μm

- **Direct Liquid Extraction Techniques**
  - LMJ/SSP: 500-800 μm
  - nano-DESI: 10-150 μm
  - SPESI: ~100 μm / 35 μm
  - MasSpec Pen

Desorption Electrospray Ionization (DESI)

DESI is a simple approach that allows ambient sampling for MS analysis. Electrosprayed aqueous droplets are directed at a surface of interest in air.

- **Spray and fluid velocity illustration and droplet-surface collision**
Hidden Messages in Paintings Displayed by DESI-MS Imaging

Experiments performed by Alan Jarmusch and Kevin Kerian
**DESI Features**

- No need or organic matrix deposition
- Suitable to the application of ambient reactions
- Non-destructive: allows further MS analysis and histochemistry
- 35 micron spatial resolution (brain tissue)
- Usually used for profiling, but can be quantitative
- High-throughput analysis (1 sec/sample)

**Intraoperative DESI Analysis**

Intraoperative DESI-MS in Surgical Suite at Indiana University School of Medicine

Jarmusch et al. Proc Natl Acad Sci U S A. 2016 Feb 9;113(6):1486-91
Alfaro et al. J Neurosurg. 2019 Jan 4;1-8

From Tissue to Chemical Reaction and Enzymatic Assay HT Analysis

**HIGH THROUGHPUT SCREENING BY DESI-MS IMAGING**

- Pathology H&E
- Micro-extraction in unmodified samples and full mass scan data

Jarmusch et al. PNAS, 2016, 113(6): 1486-1491

Partially Solvated ‘Super Reagent’: Suggested Basis for Acceleration

- $10^1$ to $10^5$ reaction acceleration is observed in droplets

**Typical solution phase reaction**
- Rate constant $10^9$ L mol$^{-1}$ sec$^{-1}$

**Typical ion/molecule reaction**
- Rate constant $6 \times 10^{11}$ L mol$^{-1}$ sec$^{-1}$

**Partial solvation**

**Droplet confinement**

Incompletely solvated reagent on the surface of a droplet acts as a highly reactive “super-reagent”, with reactivity intermediate between that in bulk solution (fully solvated) and in vacuum (unsolvated reagent).
DESI-MS High Throughput Label Free Enzymatic Assays

Acetylcholinesterase assay, relevant in drug discovery for Alzheimer’s disease as well as countermeasures against chemical warfare agents, can be performed rapidly using DESI-MS after short incubation at room temperature.

Key Points:
- Only the substrate (acetylcholine, m/z 146) and product (choline, m/z 104) must be monitored: completely label free.
- Direct analysis from the bioassay mixture (enzyme, phosphate buffer 0.1 M pH 8, 0.1% BSA) after quenching with ACN and pinning (50 nL) on a PTFE-coated substrate.

The effective analysis time is 0.3 s/sample, resulting in 7 minutes per 384-well array.

Slide credit: Nick Morato

Nano-DESI

Mouse uterine tissue

- Avoids solvent splashing though the liquid microjunction.
- Standard for quantification can be supplied with the extraction solvent at a constant rate.
- Multiple standards may be used
- Matrix effects are inferred from variations in the signal of the standard across the sample.
- Normalization to the signal of the standard compensates for matrix effects and provides relative quantification.

Alternating positive/negative mode acquisition. Spatial resolution ~10 micron
Intraoperative REIMS (iKnife)

Surgeons use the iKnife to identify cancerous tissue in real-time.
https://www.selectscience.net/editorial-articles/the-iknife-transforming-the-cancer-surgery-paradigm/?artID=47332


SpiderMass

- Designed for mobile in vivo and real-time surface analyses of biological tissues.
- Uses a fibered laser, which is tuned to excite the most intense vibrational band of water, resulting in a process termed water-assisted laser desorption/ionization (WALDI).
- The water molecules act as an endogenous matrix in a matrix-assisted laser desorption ionization (MALDI)-like scenario, leading to the desorption/ionization of biomolecules (lipids, metabolites and proteins).
- The ejected material is transferred to the mass spectrometer through an atmospheric interface and a transfer line that is several meters long.

Mass Spec Pen

- Controlled and automated delivery of a discrete water droplet to a tissue surface for efficient extraction of biomolecules.
- Applicable to intraoperative molecular analysis of lipids and metabolites for real-time diagnosis.

Zhang et al. Sci Transl Med. 2017 Sep 6;9(406):eaan3968

Direct Analysis in Real Time (DART)

- Most widely used plasma-based ambient ionization method.
- Heated metastable inert gas (e.g., helium gas) generated from a glow discharge, is used to desorb analytes present on a surface.


- Can analyze biofluids (blood, plasma, urine), medicine tablets, hair samples
- Other plasma-based methods: Atmospheric Pressure Solid Probe (ASAP), Low-Temperature Plasma probe (LTP) and Flowing Atmospheric Pressure Afterglow (FAPA).
- DART is widely used in forensics
- Has been coupled to portable mass spectrometers

SPME-TM) coupled to a portable mass spectrometer via DART

How Do I Know Which Ambient Ionization to Try for a Clinical Application?

1. Is/are **small molecule(s)** (drug, lipid, metabolite, exogenous compound)?
   - **YES**
   - **NO**

2. Did it have been already detected by an ambient method?
   - **YES**
   - **NO**

3. Is it a purified, or an abundant protein or a peptide?
   - **YES**
   - **NO**

4. Is there a method that seems appropriate?
   - **YES**
   - **NO**

5. Is there an ambient method which was used to detect similar molecules?
   - **YES**
   - **NO**

6. Do you want to invest effort in trying that?
   - **YES**
   - **NO**

7. Is this method appropriate for the throughput required?
   - **YES**
   - **NO**

8. Is this ambient source commercial?
   - **YES**
   - **NO**

9. Find a collaborator
   - **YES**
   - **NO**

10. Try to acquire evaluate feasibility with the company or through a collaboration
    - **YES**
    - **NO**
Acknowledgements

ALL AMBIENT MS INNOVATORS

Graham Cooks group
Hannah M. Brown
Nicolas M. Morato

Waters Corporation
Roy Martin
Chelsea Plummer

Bindley Bioscience Center
Bruce R. Cooper
Amber Jannasch
Yu Han-Hallett
Jackeline Franco
Uma Aryan

Livia Eberlin – UT Austin

Andy Schaber
Kameron McCree
Susan McCree
Natasha Nikolaidis
Ramaswamy Subramanian

Julia Laskin – Purdue
Classes of Molecules Detected by Ambient MS imaging

**Lipids**
Biochim Biophys Acta. 2011 Nov;1811(11):946-60

**Metabolites**
Int J Cancer. 2020 Jul 1;147(1):256-265

**Drugs (illicit and non-illicit)**

**Natural Products**

**Exogenous small molecules (warfare)**

**Peptides and Proteins**

**Permeation of Sodium Channel Modulators**
Jiao et al., Analyst. 2007 May;132(5):461-7.

**Eutectic mixture of local anesthetics (EMLA) cream**

**Basic Violet 3**

**Basic Blue 2**
Cancer Diagnosis by DESI-MS Imaging

Tissue Sections DESI-MSimaging MS lipid information 2D ion images

- **Metabolic classification of thyroid nodules** – DeHoog et al., Proc Natl Acad Sci U S A. 2019 Oct 22;116(43):21401-21408
- **Squamous cell carcinomas** – Wollman et al., Analyst. 2017 Aug 21;142(17):3250-3260
- **Prostate Cancer** – Banerjee et al. Proc Natl Acad Sci U S A. 2017 Mar 28;114(13):3334-3339; Eberlin et al., Anal Chem. 2010 May 1;82(9):3430-4
- **Human bladder carcinoma** – Dill et al., Chemistry. 2011 Mar 1;17(10):2897-902

**DESI-MS³ Intraoperative Assessment of IDH Mutation in Gliomas**

- Summed MS³ fragment ion intensities (m/z 85 + m/z 101) produced by sequential dissociation of 2HG

- **Isocitrate dehydrogenase (IDH) mutation status is a strong prognostic marker and is used in glioma subtyping.**
- **DESI-MS intensity of 2HG, the oncometabolic product of the IDH mutation, strongly correlates with IDH mutation status.**
- **The intensity of the 2HG fragment ions are used to diagnose glioma specimens as IDH Mutant or IDH Wild-type.**
3D DESI-MS lipid imaging in a xenograft model of glioblastoma

- Visualization of lipid heterogeneity in lipid a glioblastoma xenograft tumor
- Used an automatic slide loader automates 3D imaging for high sample-throughput
- Combination of chemical and morphological information allowed the association of different lipid species with hypoxic and viable tissue within the tumor.

- m/z 428 and m/z 400 have been identified as stearoylcarnitine and palmitoylcarnitine
- Carbonic anhydrase-9: Marker of hypoxic signaling

Protein Analysis by DESI-MS

Concentration-dependent improvement in protein signal intensity when micromolar to low millimolar concentrations of serine is combined with formic acid in DESI spray.

Protein Analysis by DESI-MS Using Heated Sprayer and Ion Mobility

The combination of DESI with ion mobility can improve the signal to noise and allow the separation of highly complex overlapping signals.

Liver tissue

(a) high velocity region, multiply charged peptides and small proteins;
(b) haemoglobin trend line
(c) solvent peaks, remaining lipids and other small molecules (singly charged)

Mobility extracted endogenous peptide peaks with corresponding distributions (sum of isotopes)
DESI-MS Workflow – Samples and Surfaces

HISTOLOGICAL SECTIONS

Mouse preimplantation embryos

SAMPLES IN NATURA PLACED ON SURFACES OR TRANSFERRED TO SURFACES

Cultured cells


DESI-MS Workflow – Direct Sampling

 skin surface sample taken by imprinting with glass slide

Sample taken from Visitor’s forearm

Most intense ions separated by m/z 22 interval, which is indicative of PEG. No evidence of silver adducts based on isotope patterns.

DESI-MS in the positive ion mode using acetonitrile doped with Ag2NO3 6ppm as the spray solvent

Unpublished data
Sample taken from Lab member forehead skin

Most abundant ion is squalene. Other ions are mostly tryacylglycerols (TAGs).

Examples of Surfaces for DESI-MS
- Glass or PTFE
- Pharmaceutical Tablet
- Tissue Samples
- Plant Material
- Chromatographic plate after separation
- Mesh
- Liquid samples from a capillary

Unpublished data

*Sunscreen/cosmetic composition may have influenced (tinted sunscreen)

Reproducibility of Imaging analysis of human cancer tissue using DESI-MS

- Repeatability and reproducibility for measurements of lipid intensities in tissue sections were 22% and 18%, respectively.
- The reproducibility of DESI-MS is appropriate for accurate lipidomic profiling studies of human tissue for meaningful inter-sample comparison of various disease states such as cancer.
- DESI-MS not only has the added advantage of spatially and histologically resolved data acquisition, it also has acceptable levels of reproducibility for the purposes of lipidomic profiling.

### DESI Spray Stability in the Negative Ion Mode Is Dependent on Relative Humidity

- Under low-humidity conditions (~15–30%), negative ion mode DESI mass spectra were prone to spray artifacts.
- Stable and typical mass spectra were obtained in RH levels of 35–60%.
- Signal instability could be due to corona discharges occurring at the DESI spray under low-humidity conditions.


### DESI-MS Imaging Resolution

A combination of smaller step-size with shorter MS scan-times, slower imaging scan-rates, plus the use of morphologically friendly solvent systems and relatively low solvent flow rates enabled the improvement of DESI imaging spatial resolution on mouse brain cerebellum tissue.

Improved Resolution with the use of a particular combination of solvent system and with smaller step-sizes.

1.0 μL/min of DMF/EtOH (1:2)

DESI-MS Imaging Resolution — Scan time and solvent flow

1.5 μL/min of DMF/EtOH (1:2)

Improved Spatial Resolution in a mouse ovary chemical image

- Solvent composition
- Solvent flow rate
- Probe step-size
- Scan-time

Shorter scan-rate allows ions from one point to be rapidly scanned before the subsequent point of the image is sampled.

DESI Spray Technological Evolution — Early days

Capillary cutting and checking in a microscope

Putting together pieces

0.4mm ferrule with outer capillary

0.3mm ferrule with inner capillary

Union Tee

N₂ gas entry
Intense progress in analytical instrumentation for DESI-MS imaging: from home-built to commercial, automated systems with dedicated software.

Electrospray design within ionKey high-pressure microfluidic device was incorporated to improve DESI sprayer. Briefly, stainless steel emitter recessed behind critical orifice, nebulization gas sheaths primary solvent to create liquid jet.

Positive ion mode DESI MS images of a PC lipid in liver acquired at (a) 100 µm and 20 µm pixel size showing hepatic lobules; the scale bar used is 500 µm for both images, (b) a composite negative ion mode image of m/z 195.05 (blue) and 293.21 (red) ions imaged at 25 µm pixel size.

Positive ion mode DESI MS images of sagittal section of rat brain section performed at 80 µm pixel size. (a) is composite image of ions in (b) cholesterol, (c) a PE lipid, and (d) PC(34:1); (e) is zoomed in version of the cholesterol ion.

Shresta et al., ABRF 2020 poster
**Pixel, Time of Data Acquisition and Speed calculation**

To calculate the number of pixels in each line:

\[ \frac{78,500 \text{ µm (x distance)}}{350 \text{ µm (step size)}} = 224.3 \text{ Pixels} \]

Time of data acquisition for each line:

\[ 224.3 \times 0.08 = 17.94 \text{ sec/60 = 0.3 min} \]

DESI stage speed: \[ \frac{78,500}{17.94} = 4376 \text{ µm/sec} \]

**Parameters for DESI Sprayer Tuning**

Stable spray: microdroplets (1-10 µm) accelerated by a nebulizing sheath gas

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage (kV)</td>
<td>1</td>
<td>5</td>
<td>3.9</td>
<td>26</td>
</tr>
<tr>
<td>Solvent flow rate (µL/min)</td>
<td>1.5</td>
<td>25</td>
<td>5.6</td>
<td>27</td>
</tr>
<tr>
<td>Incident angle (°)</td>
<td>35</td>
<td>90</td>
<td>56</td>
<td>24</td>
</tr>
<tr>
<td>Sprayer-to-surface distance (mm)</td>
<td>1</td>
<td>5</td>
<td>2.0</td>
<td>22</td>
</tr>
<tr>
<td>Gas pressure (psi)</td>
<td>100</td>
<td>261</td>
<td>136</td>
<td>18</td>
</tr>
</tbody>
</table>

Figure graciously provided by Waters Corporation (Bindesh Shresta)

**Reference**

**Overall DESI-MS Imaging Workflow (Synapt Platform)**

1. Optimize DESI Spray (Once for a sample type. Issue with multiple users)
2. Check instrument calibration and sensitivity (brain tissue)
3. Take a Picture of the slide and upload it at the HDI software
4. Select region to be imaged
5. Define resolution and speed, if want to use IMS and its parameters
6. Save project, make sure the solvent amount in the syringe is enough for all data acquisition time
7. Open MassLynx, import project worklist, Start data acquisition
8. Check if the sprayer is starting and stopping to acquire data for each line in the expected positions
9. When the spray starts hitting the sample, check if mass spectra have good quality
10. Process the data (select m/z range and number of peaks of interest)

**Visualization of Ion Distribution and Data Analysis**

- Normalization by the TIC
- Plotting of individual ion images
- Subtraction of regions of interest (ROI)
- Ion image overlay
- HDI allows ion mobility data visualization

Check the Dec 3rd slides from Michelle Reyzer

![Image of Ion Distribution](https://ms-imaging.org/wp/biomap/)

**Links**

- CARDINAL SOFTWARE: [https://cardinalmsi.org/](https://cardinalmsi.org/)
- Waters HDI Software: [https://ms-imaging.org/wp/biomap/](https://ms-imaging.org/wp/biomap/)
**DESI-MS Profiling Workflow – Microscopic Samples**

1mm or 0.04 in
Blastocyst: 100-200 cells

**Lipids typically detected in a DESI-MS mass spectra of a single bovine embryo**

<table>
<thead>
<tr>
<th>m/z</th>
<th>Species</th>
<th>m/z</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>255.4</td>
<td>FA (16:0)</td>
<td>788.4</td>
<td>PS (36:1)</td>
</tr>
<tr>
<td>279.4</td>
<td>FA (18:2)</td>
<td>794.3</td>
<td>PC (34:1) + Cl</td>
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<tr>
<td>281.4</td>
<td>FA (18:1)</td>
<td>810.5</td>
<td>PS (38:4)</td>
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<td>283.4</td>
<td>FA (18:0)</td>
<td>816.3</td>
<td>PS (38:1)</td>
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<td>303.4</td>
<td>FA (20:4)</td>
<td>820.4</td>
<td>PC (36:2) + Cl</td>
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<tr>
<td>327.3</td>
<td>FA (22:6)</td>
<td>822.5</td>
<td>PC (36:1) + Cl</td>
</tr>
</tbody>
</table>

Chemical Reaction Screening by DESI-Imaging (DARPA Make-it)

Chemical Science 2018 Jan 4;9(6):1647-1653
Chemistry 2018 Jul 5;24(38):9546-9554
Chemical Engineering Science, 2019, 195: 1010-20

DESI-MS Imaging Workflow – High Throughput Screening
Software and Hardware Modifications for DESI Reaction Screening

ElveFlow

Illustration of spot-by-spot DESI-MS data collection

Yes/No Output of 6,144 spot plate

Repository/Interactive view

CHRIS Software

High-Throughput Label-Free Enzymatic Assays Using Desorption Electrospray-Ionization Mass Spectrometry

- 0.3 s per sample.
- Study of the acetylcholinesterase assay: kinetic parameter determination, rapid inhibitor screening, IC50 and Kᵢ, as well as inhibition–reactivation assays.

ACHE reaction (1 mm ACh). Inset shows the calibrated linear region (R² 0.995) used to calculate v0. Each data point represents the average of 8 replicates. The CV of all points is below 15 %.

Michaelis–Menten plot of the ACHE reaction, relating v0 and substrate concentration. 18 independent progress curves, acquired on different days, were used to build this plot. The CV of all data points is below 5 %. Adjusted R² of the fit is 0.998.


DESI-MS Imaging Summary and Perspectives in Clinical Applications

**Tissue Imaging**
- Suitable for clinical diagnosis in diverse instances
- Protein detection
- Combination with ion mobility increases chemical information

**Intraoperative Diagnosis**
- Mini mass spectrometers in the operating rooms
- Automated data analysis
- Multi-site study

**Chemical Reaction Screening**
- Reliable HT (10,000 samples/hour) guidance to flow synthesis and to enzymatic assays
- Numerous chemistries can be assayed, automated
- Precise spot location, modularity of the system allows integration of new instrumentation
- Drug discovery/clinical trial (Generic drug Synthesis & Sonogashira Coupling)

Acknowledgements

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Uma Aryal

Andy Schaber
Kameron McCreery
Susan McCreery
Natasha Nikolaidis
Ramaswamy Subramanian

Livia Eberlin – UT Austin
Bringing IMS and AMS into the clinic: opportunities and challenges

1. Tackling Diagnostic Blind Spots
   A. Lipids
   B. Cancer metabolism
   C. Rapid Results
   D. Infectious Diseases
   E. Imaging drugs

2. Wrap-up
   A. Considerations for bringing new mass spectrometry techniques into the clinical arena
   B. Final thoughts and advice
Tackling Diagnostic “Blind Spots” using mass spectrometry:

Lipids

Clinical Case 1
Lipids in brain cancer


Lipids in thyroid cancer

Lipids in prostate cancer


Lipids in bladder cancer

Lipids in breast cancer

- Similar methods also employed in renal, lung and other cancers as well
- These are complex mixtures of cells, is there a way to look at pure cells to better understand the biology


Conventional methods to characterize lipidomic profiles in vitro

Cell Culture → Washing → Harvesting (scraping, trypsinizing) → Centrifugation → MS/MS → HPLC → Liquid-Liquid Extraction
In vitro liquid extraction surface analysis (ivLESA)

Adherent cells (96-well plate) Aspirate culture media

In vitro liquid extraction surface analysis (ivLESA)

Nano-electrospray ionization

Intensi m/z MS analysis

ivLESA “up close”

Basu SS, Randall EC, Regan MS, Lopez BGC, Clark AR, Schmitt ND, Agar JN, Dillon DA, Agar NHR. In vitro Liquid Extraction Surface Analysis Mass Spectrometry (ivLESA-MS) for Direct Metabolic Analysis of Adherent Cells in Culture. Analytical Chemistry 2018
Breast cancer cell lines demonstrate different lipidomic signatures

<table>
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<tr>
<th>Cell Line</th>
<th>n</th>
<th>R</th>
<th>PR</th>
<th>Her-2</th>
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<tr>
<td>MCF-7</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>ZR-75-1</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>MDA-MB-453</td>
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<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

MDA-MB-231 cells show higher relative abundance of particular lipids

Basu et al. Analytical Chemistry 2018
### HRMS using ESI-FT-ICR MS in MDA-MB-231 cells

<table>
<thead>
<tr>
<th>m/z meas</th>
<th>m/z calc</th>
<th>Molecular Formula</th>
<th>Adduct</th>
<th>Molecular assignment</th>
<th>Delta (ppm)</th>
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<tr>
<td>572.48120</td>
<td>572.48150</td>
<td>C_{34}H_{67}NO_{3}</td>
<td>M+Cl^-</td>
<td>Ceramide (d18:1/16:0)</td>
<td>0.5</td>
</tr>
<tr>
<td>682.59060</td>
<td>682.59105</td>
<td>C_{42}H_{81}NO_{3}</td>
<td>M+Cl^-</td>
<td>Ceramide (d18:1/24:0) (15z)</td>
<td>0.7</td>
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<tr>
<td>684.60623</td>
<td>684.60670</td>
<td>C_{42}H_{83}NO_{3}</td>
<td>M+Cl^-</td>
<td>Ceramide (d18:0/24:0)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

C16 Ceramide

C24 Ceramide

C24:1 Ceramide

---

### Ceramides

- **Phosphoglycerides:** Glycerol backbone
- **Ceramides:** Serine backbone
Tackling Diagnostic “Blind Spots” using mass spectrometry:

Cancer metabolism

Clinical Case 1

IDH1

Krebs Cycle
IDH mutations have diagnostic and prognostic importance in glioma


2-HG can be differentiated from αKG by MS

**2-hydroxyglutaric acid (2-HG)**
C₇H₆O₅
148.11 g mol⁻¹

**Alpha-ketoglutarate acid**
C₅H₆O₅
146.11 g mol⁻¹

(147.11 in negative mode)
2-Hydroxyglutarate (2-HG) Detection by DESI-MS

Confirmation of Metabolite Identity

Banked Glioma Specimens Analyzed

Sample ID | Diagnosis | % tumor | IDH Wild Type | IDH1 Mutated Sample
--- | --- | --- | --- | ---
Low tumor cell concentration
G11 | Wild type | 10 | positive | 72 | positive
G12 | Wild type | 20 | positive | 82 | positive
G13 | Wild type | 5 | positive | 45 | negative
G14 | Wild type | 5 | positive | 35 | negative
G15 | Wild type | 10 | positive | 47 | negative
G16 | Wild type | 15 | positive | 46 | negative
G17 | Wild type | 20 | positive | 79 | positive
G18 | Wild type | 10 | negative | 7 | negative
G19 | Wild type | 0 | negative | 178 | negative
High tumor cell concentration
G21 | Wild type | 30 | positive | 274 | positive
G22 | Wild type | 50 | positive | 243 | positive
G23 | Wild type | 10 | negative | 79 | negative
G24 | Wild type | 10 | positive | 48 | positive

G23, G28, G33

Genetic analysis reveals another diagnostic blindspot

Tackling Diagnostic “Blind Spots” using mass spectrometry:

Rapid results
Faster inter-operative guidance needed

Image courtesy of Lab Tests Online: Anatomic

Advanced Multimodality Image-Guided Operating Suite (AMIGO)

- MRI
- PET/CT
- X-Ray/Dyna CT
- Ultrasound
- Navigation
- Optical Imaging

National Biomedical Technology Research Resource for Image-Guided Therapy
NIH (P41EB015898) and Brigham and Women’s Hospital
Intraoperative MS at BWH

Ambient MS allows measurements performed directly on sample

Sample Collection → Sample Processing → Liquid Chromatography → Electrospray Ionization → Mass Spectrometry

Ambient ionization allow real time or near real time analysis
Interchangeable ambient ionization source

DESI

LMJ-SSP

Interchangeable interface

Prosolia Inc.

Pioneered for neurosurgical applications

General Intra-operative MS workflow

Specimen Collection
Surgical Resection

Minimal Sample Prep
Smash/Squash Prep

Extraction: Ionization
Ambient Ionization

Molecular Analysis
Ion trap MS

Concordance of 2HG Signal with Tumor Cell Concentration
Intra-operative workflow and specific challenges with fresh breast tissue

**Specimen Collection and Allocation**
- Lumpectomy, margins
- Subject enrollment

* Tumor and normal
  Only if tumor is >1.5 cm

**Sample Preparation**
- Smash/Squash Prep

**Analyte Extraction**
- Liquid microjunction Surface sampling probe (LMJ-SSP)

**MS Analysis**
- Ion trap MS

No standard biomarker for breast cancer (like 2HG for glioma)

**Surgical Breast cases in AMIGO**

<table>
<thead>
<tr>
<th>Case</th>
<th>Invasive Carcinoma Type</th>
<th>Tumor size (cm)</th>
<th>Overall Histological Grade</th>
<th>ER</th>
<th>PR</th>
<th>Her-2 Margin</th>
<th>Normal Tumor</th>
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<tbody>
<tr>
<td>1</td>
<td>Mixed</td>
<td>1.6</td>
<td>Poor</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>2</td>
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<td>1.5</td>
<td>Moderate</td>
<td>+</td>
<td>+</td>
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<td>6 1</td>
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<td>+</td>
<td>+</td>
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<td>1.4</td>
<td>Well</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6 1 2</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>6</td>
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<td>1.6</td>
<td>Well</td>
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<td>+</td>
<td>-</td>
<td>6 2 1</td>
</tr>
<tr>
<td>7</td>
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<td>Poor</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6 1 1</td>
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<tr>
<td>8</td>
<td>Ductal</td>
<td>2.1</td>
<td>Poor</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6 1 1</td>
</tr>
<tr>
<td>9</td>
<td>Ductal</td>
<td>0.8; 0.5</td>
<td>Well</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>6 1</td>
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<td>10</td>
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<td>0.9</td>
<td>Well</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>6 1</td>
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<tr>
<td>12</td>
<td>Ductal with spindle features</td>
<td>0.6</td>
<td>Moderate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6 1 2</td>
</tr>
<tr>
<td>13</td>
<td>Ductal</td>
<td>1.9</td>
<td>Poor</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>6 1 1</td>
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<td>Moderate</td>
<td>+</td>
<td>+</td>
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<td>6 1 1</td>
</tr>
<tr>
<td>15</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>Well</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>6</td>
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<td>+</td>
<td>+</td>
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<td>6</td>
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<td>19</td>
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<td>6</td>
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<td>Moderate</td>
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<td>+</td>
<td>-</td>
<td>6 2 1</td>
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<tr>
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<td>Moderate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>6 1</td>
</tr>
</tbody>
</table>
Representative mass spectra demonstrating differences between normal and tumor specimens

Normal
Tumor

Tumor demonstrates more abundant phospholipid profile

Representative Normal (Case 13)

Stimulated Raman Spectroscopy (SRS)
Second Harmonic Generation (SHG)
Lipids
Collagen
Representative Tumor (Case 13)

Stimulated Raman Spectroscopy (SRS):
- Lipids
- Collagen

Second Harmonic Generation (SHG):

MS Data analysis

Raw MS data
- Entered into Matlab
  - Aligned (msalign)

Class labeling

Visualization
- Dimensionality reduction using t-distributed stochastic neighbor embedding (t-SNE)
- Significant analysis of microarray (SAM)
- Candidate biomarkers

Significance analysis of microarrays (SAM)
Significance analysis of microarrays (SAM)

5 metabolites demonstrate FDR < 0.00001

Tumor Normal

t-SNE visualization

Colored using m/z = 767.6

Colored using m/z = 883.7

High

Low
Can we bring MALDI MSI to the frozen section room?

Conventional MALDI MSI Workflow:
Three time-intensive steps

1. Cryomounted frozen tissue section
2. Matrix application (and drying)
3. Slide scanning and image registration
4. MALDI MSI Data Acquisition
MALDI MSI is powerful...but can we go faster?

Conventional MALDI MSI:
- No pre-treatment
- No pre-application
- No imaging
- Preparation to acquisition time: 30-60 min

Rapid MALDI MSI:
- Matrix pre-coating ITO slide
- No matrix application and drying
- No image registration
- 10 kHz laser
- Preparation to acquisition time: 2-4 min

Matrix pre-application:
- 40 mg/mL 2,4,6-Trihydroxyacetophenone (THAP)
- Solvent: 9:1 acetonitrile: water, sonicated 10 min

Images showing 1 pass, 2 passes, 4 passes, and 6 passes of matrix application.
Pre-templated slide and high frequency laser

Rapid MALDI MSI Workflow: Significant time savings

1. Cryomounted frozen tissue section
2. Matrix application (and drying)
3. Slide scanning and image registration
4. MALDI MSI Data Acquisition

<table>
<thead>
<tr>
<th>Spatial Resolution</th>
<th>Total Acquisition Time</th>
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<tbody>
<tr>
<td>Single gradient</td>
<td></td>
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<tr>
<td>20 μm</td>
<td>3 min</td>
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<td>100 μm</td>
<td>18 min</td>
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<td>50 μm</td>
<td>25 min</td>
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</tr>
<tr>
<td>50 μm</td>
<td>8 min</td>
</tr>
<tr>
<td>20 μm</td>
<td>6 min</td>
</tr>
</tbody>
</table>

10 - 60 min
10 - 15 min
3 - 5 min
Higher signal intensity where tissue is ablated/penetrated by laser

MALDI MSI (m/z 888.7) Reflective bright field microscopy

M5 defocus laser with high detector gain (3048 volts)

Optimizing laser parameters improved signal

Elizabeth Randall

Basu, Regan et al. NPJ Precis Oncol. 2019; 3:17
Rapid MALDI MSI identifies unique signatures and biomolecules in tissue

(A) Healthy mouse brain
(B) Mouse brain with human glioblastoma (PDX)
(C) Normal human breast tissue
(D) Human breast carcinoma
(E) Human glioblastoma

Walid Abdelmoula
Non-linear registration used to fuse MALDI and histology images

Rapid MALDI MSI for Surgical Pathology: Summary and future directions

Basu, Regan et al. NPJ Precis Oncol. 2019; 3:17
Tackling Diagnostic “Blind Spots” using mass spectrometry:

Infectious diseases

Clinical Case 2
Metal Oxide Laser Ionization (MOLI)

- Background: Cerium oxide (CeO₂) is a rare earth metal which showing promise for metal oxide laser ionization (MOLI) MS

MOLI-MS for bacterial lipid analysis

Table 6. Percent accuracy of biotype bacterial ID and CoA-catalyzed MOLI fatty acid analysis.
Can we use MOLI-MS for mass spectrometry imaging (MOLI MSI)?

First, confirm MOLI MS works on our instrumentation
Overlaying CeO$_2$ matrix was needed for optimal extraction and ionization

MOLI-MSI reveals fatty acid pool differences throughout mouse brain
MOLI-MSI reveals fatty acid pool differences in PDX tumors

MOLI-MSI using CeO₂ of "pseudo-infection" model

Tackling Diagnostic “Blind Spots” using mass spectrometry:

Measuring drugs in tissue

Clinical case 3

Crystals
High resolution MSI using MALDI-FTICR of tissue fragments

Unstained tissue
Morphine 286.144 +/- 0.001
Morphine-D3 (ISTD) 289.163 m/z +/- 0.001

LESA-MS/MS of FFPE tissue confirms expected CID morphine product ions
Blood-Brain Barrier Imaging

Heme as a Vasculature Marker

**BKM120 Crosses the BBB**

BKM120 at therapeutic dosage
U87 Tumor
4h Treatment
100µm resolution


---

**Inhibitor RAF265 on a physiologically relevant model of PLGA**

Claire Sauvageot, Mark Marchionni, Erin Davis

---

\[
p = 0.000212
\]

\[
p = 0.216
\]
A paradox: RAF265 appears to target the tumor but the response is poor.

Higher resolution resolves the paradox: RAF265 actually has poor BBB penetrance.

IHC of Blood Vessels
Endothelial Marker CD 31


Significant variability of drug concentration within tissue

5E 797 ng/g
5E low

5E high

5E 537 ng/g

5E 1540 ng/g

R² = 0.9881

y = 0.8243x
Bringing IMS and AMS into the clinic: opportunities and challenges

1. Tackling Diagnostic Blind Spots
   A. Lipids
   B. Cancer metabolism
   C. Rapid Results
   D. Infectious Diseases
   E. Imaging drugs

2. Wrap-up
   A. Considerations for bringing new mass spectrometry techniques into the clinical arena
   B. Final thoughts and advice

Consideration 1: Going from research to clinical care
Utilization of tissue mimetics and stable isotope internal standards to improve quantification of 2-HG


Consideration 2: In clinical world, pre-analytics is key

Experiment from pathology rotation in medical school

Control
(time 0 h)

Overnight incubation in:

- RPMI
- Air
- Saline

Metabolomics: the restaurant analogy

Genomics (Ingredients) → Proteomics (Menu) → Metabolomics (Taste)
**Metabolomics: The good...**

Relatively lower complexity of analysis

~30,000 genes → ~100,000 proteins (with PTMs) → ~7000 metabolites

Analytical turnaround time

Weeks, months? → Days → Hours (DESI-MS: in real time!)

**Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL. Chem Soc Rev. 2011:40;387.**

---

**Metabolomics: The bad...**

Relatively expensive technologies

Next generation sequencing → IHC → Mass spectrometry

 Fewer absolutes in interpretation

+/− Presence of mutations → +/− Presence of protein expression → Relative levels of large panel of different metabolites

**MSACL Connect: Imaging 101: Clinical Studies using Imaging MS ©2020 Sankha Basu, Michelle Reyzer, Christina Ferreira**
Metabolomics: The ugly...

Genomics → Proteomics → Metabolomics

Highly variable analyte stability

- Weeks to years
- To decades?
- Hours to days
- Minutes (Maybe Seconds!)

Sample collection, handling and processing are critical when planning or designing a metabolomics study.

Metabolomics on pathology specimens: Pre-analytical considerations

- Formalin
- Likely needs to be frozen relatively quickly
- OCT
- Dehydration
- Methanol, Ethanol, Xylenes, etc.

Sample collection, handling and processing are critical when planning or designing a metabolomics study.
Consideration 3:
Spread the word - talk to your friends!!

Identification of Bacteria Using Mass Spectrometry

John P. Ackley

Department of Laboratory Medicine, The Johns Hopkins Medical Institutions, Baltimore, Md. 21205.

Catherine Fenselau

Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205.

Colindent post-mortem mass spectrometry has been applied to the identification of certain pathogenic bacterial species. Other methods of bacterial identification, such as the use of polymicrobial cultures or the tox disagreement of these specimens, are less accurate because of the high sensitivity of mass spectrometry. The spectra obtained from genomic probes of these probes can be used to identify bacteria based on their mass spectrometry profiles. The approach to detecting potential pathogens by mass spectrometry is described.

Catherine Fenselau

Consideration 4:
Question dictates the tool

Swiner et al. Analytical Chem 2019

MSACL Connect: Imaging 101: Clinical Studies using Imaging MS ©2020 Sankha Basu, Michelle Reyzer, Christina Ferreira
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- Madison McMinn
- Armen Changelian
- Isaiah Norton
- Frank Lu

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- Alexandra Golby
- Ian Dunn
- Edward Laws
- Melissa Mallory

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- Ron Kikinis
- Clare Tempany

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- Sandro Santagata
- Keith Ligon
- Kristi Gill

Northeastern University
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- Mark Kieran
- Rosalind Segal

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- Miriam ElNaggar

Bruker Daltonics
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- Victor Fursey

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- R01 Mass Spectrometry for Intraoperative Tissue Characterization in Breast-Conserving Therapy
- T32 HL007627 (Pathology, BWH)
- Robbins Award (Pathology, BWH)
- Brain Science Foundation
- Daniel E. Ponton Fund for the Neurosciences
- Pediatric Low-Grade Astrocytoma Program, DFCI
- P41 National Center for Image Guided Therapy (NCIGT)
- U54 Physical Sciences Oncology Center (Mayo/MIT/BWH)