WEBINAR

Microfluidic precision autostainer for fast multiplexed TSA-based immunofluorescence
Webinar agenda

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Microfluidic technology & LabSat® autostainer

Automated TSA-based multiplexing: tonsil optimization

Easy protocol transfer to a specific cancer type.
Case study: NSCLC
Chip with microfluidic channels network for reagents delivery.

Creates a closed chamber over the tissue sample.

Clamped against a standard slide.
Microfluidics for tissue analytics

Fast Fluidic Exchange Technology

PRESSURE CONTROLLED
The reagents are pushed into the microfluidic channels, filling the chamber almost instantaneously.

TEMPERATURE CONTROLLED
A heating element is present under the slide providing great control of temperature cycles during protocols.

CLOSED CHAMBER
A microfluidic chip is clamped against the slide creating a low-volume hermetic chamber.
Microfluidics for tissue analytics

Fast Fluidic Exchange Technology

- Fast staining time
- Uniform staining over the tissue
- Rapid and high-temperature epitope retrieval
- No reagent evaporation
- Tissue preservation
Technology: Publications

“Microfluidic processor allows rapid HER2 immunohistochemistry of breast carcinomas and significantly reduces ambiguous (2+) read-outs.”


“Continuous quantification of HER2 expression by microfluidic precision immunofluorescence estimates HER2 gene amplification in breast cancer”

D.G. Dupouy et al., Scientific Reports no. 6, pp. 20277, 2016

- MTP-score concordant with HER2 FISH
- 90% reduction of ambiguous HER2 cases with MTP
- MTP-based HER2 IF correlates with the HER2 gene copy number
Automated microfluidic stainer

- Buffers reservoirs
- Distribution System
- Waste
- Reagents reservoirs
- Slide
- Staining Chamber

LabSat® Research
Why multiplexing?

**Personalized medicine:** The number of biomarker tests increases as researchers seek clinically relevant markers to develop more precise diagnostic tests.

**Spatial and morphological context:** Need for simultaneous detection of multiple markers in their morphological context.

**Immunophenotyping:** There is an increasing trend to understand the complexity of the tumor microenvironment.
Tyramide Signal Amplification (TSA)

Working principle
TSA-based multiplexing on LabSat®

Use case: Opal® Kit

LabSat® Research + Opal® TSA Kit → Multiplexed IF
6 markers + DAPI

Lunaphore technologies
AKOYA BIOSCIENCES®
Opal® technology

Opal® 7-Color IHC Kit
7-colors TSA detection kit

- 6 TSA fluorophores of different wavelengths
  - Opal 520
  - Opal 540
  - Opal 570
  - Opal 620
  - Opal 650
  - Opal 690

- Counterstaining: DAPI

- Secondary: Mouse + Rabbit polymer HRP

- 6-plex / 7-colors assay
Objective

Develop an automatized full 6-plex with Opal® kit on LabSat®

- Heat-induced epitope retrieval (HIER)
- Protein blocking
- Primary antibody
- Enzyme-linked secondary antibody
- Tyramide signal amplification
- Antibodies strip-off
- Counterstaining

LabSat® Research

Repeat 6x

- Mounting
- Coverslipping
- Imaging

• Deparaffinization
• Rehydration
Automated TSA-based MUX optimization

4 axes of technology developments:

1. Single-plex staining
   - Single marker optimization
   - Repeatability study

2. Uniformity optimization
   - Uniformity assessment (performed with CD20 on tonsil tissue)
   - Repeatability study

3. Antibody elution
   - Antibody stripping efficiency for all markers
   - Assessment of elution impact on tissue morphology

4. Multiplexing
   - Combine all single-plex markers sequentially with elution steps in between
   - Perform full multiplex staining on LabSat®

Optimization tissue: **FFPE human Tonsil**

Selected markers and related colours (TSA):

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>FoxP3</td>
<td>Opal 570</td>
</tr>
<tr>
<td>PDL1</td>
<td>Opal 520</td>
</tr>
<tr>
<td>PD-1</td>
<td>Opal 690</td>
</tr>
<tr>
<td>CD68</td>
<td>Opal 620</td>
</tr>
<tr>
<td>CD8</td>
<td>Opal 540</td>
</tr>
<tr>
<td>PanCK</td>
<td>Opal 650</td>
</tr>
<tr>
<td>Counterstain</td>
<td>DAPI</td>
</tr>
</tbody>
</table>
Single-plex optimization
Single-plex optimization

LabSat® protocol optimization: staining
Each step is optimized to reduce execution while ensuring staining quality

- Antigen retrieval
  Temperature and pressure settings
- Reagents incubation
  Temperature, duration, and flow optimization
- Primary antibody and substrate
  Titration of antibodies and Opal® reagents

Image acquisition and quantification with Mantra™ platform
- InForm™ software used for image analysis
Image analysis

Spectral unmixing:
Process of decomposing the spectral signature of a mixed signal into a set of constituents with their corresponding weight

- In every cell, signal intensity is calculated for each constituent
- Intensity is normalized by exposure
Single-plex optimization: results

For each marker (single-plex), a bright, specific signal is obtained, with high signal-to-background ratio.

Tissue stained: tonsil (FFPE)

<table>
<thead>
<tr>
<th></th>
<th>FOXP3</th>
<th>PD-L1</th>
<th>PD-1</th>
<th>CD68</th>
<th>CD8</th>
<th>CK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image*</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Mean NI**</td>
<td>7.1</td>
<td>3.7</td>
<td>7.5</td>
<td>8.5</td>
<td>6.2</td>
<td>12.3</td>
</tr>
</tbody>
</table>

* Software-reconstructed brightfield view (Pathology view, InForm™, AKOYA)
** Mean Normalized Intensity computed with InForm™ on 3 ROI per slide
Single-plex optimization: results

Protocol variability assessment on 9 slides, tonsil tissue (FFPE)

The single-plex reproducibility study showed less than 15% of signal variability, underlining the effective automation of protocols of the system.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Slide 1</th>
<th>Slide 2</th>
<th>Slide 3</th>
<th>Slide 4</th>
<th>Slide 5</th>
<th>Slide 6</th>
<th>Slide 7</th>
<th>Slide 8</th>
<th>Slide 9</th>
<th>CV*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td>13.5%</td>
</tr>
<tr>
<td>PDL1</td>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
<td>10%</td>
</tr>
<tr>
<td>PD1</td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
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<td><img src="image24.png" alt="Image" /></td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td>9.9%</td>
</tr>
<tr>
<td>CD68</td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
<td><img src="image33.png" alt="Image" /></td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
<td>8%</td>
</tr>
<tr>
<td>CD8</td>
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<td><img src="image38.png" alt="Image" /></td>
<td><img src="image39.png" alt="Image" /></td>
<td><img src="image40.png" alt="Image" /></td>
<td><img src="image41.png" alt="Image" /></td>
<td><img src="image42.png" alt="Image" /></td>
<td><img src="image43.png" alt="Image" /></td>
<td><img src="image44.png" alt="Image" /></td>
<td><img src="image45.png" alt="Image" /></td>
<td>7%</td>
</tr>
<tr>
<td>CK</td>
<td><img src="image46.png" alt="Image" /></td>
<td><img src="image47.png" alt="Image" /></td>
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<td><img src="image50.png" alt="Image" /></td>
<td><img src="image51.png" alt="Image" /></td>
<td><img src="image52.png" alt="Image" /></td>
<td><img src="image53.png" alt="Image" /></td>
<td><img src="image54.png" alt="Image" /></td>
<td>7%</td>
</tr>
</tbody>
</table>

*Coefficient of variation of signal among the 9 stained slides, based on InForm® or Fiji (for CD68) analysis.
Uniformity
Uniformity optimization

Signal gradient issues with standard incubation

Solution: Dynamic incubation with "oscillating flow"

Dynamic incubation reduces signal gradient
Uniformity: Results

Static incubation vs. Dynamic incubation

**Standard incubation**

![Image of standard incubation](image)

<table>
<thead>
<tr>
<th>ROI</th>
<th>Signal quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
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<tr>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Signal drop over 1cm: 30%

**Dynamic incubation**

![Image of dynamic incubation](image)

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<td>4</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Signal drop over 1cm: 6%

*Intensity normalized by exposure time, averaged on 20 brightest cells per ROI*
Antibody elution
Elution optimization

**Elution step**
Removal of the primary-secondary antibodies complexes on the tissue after each marker detection

**Assessment method**
Compare signal on reference slide and eluted slide

Reference slide
- Antigen retrieval
- AbI
- AbII
- TSA

Eluted slide
- Antigen retrieval
- AbI
- AbII
- Elution (heating)
- Elution (heating)
- TSA

**Imaging + Quantification**

**Elution efficiency**
\[ E_{elution} = \frac{\text{Signal}_{Ref} - \text{Signal}_{Elu}}{\text{Signal}_{Ref}} \times 100 \]
## Elution study: Antibody strip-off efficiency

The elution method in LabSat® consists of a heating cycle after each marker detection.

<table>
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<tbody>
<tr>
<td>Reference slide</td>
<td><img src="reference-slide.png" alt="Image" /></td>
<td><img src="reference-slide.png" alt="Image" /></td>
<td><img src="reference-slide.png" alt="Image" /></td>
<td><img src="reference-slide.png" alt="Image" /></td>
<td><img src="reference-slide.png" alt="Image" /></td>
<td><img src="reference-slide.png" alt="Image" /></td>
</tr>
<tr>
<td>Eluted slide</td>
<td><img src="eluted-slide.png" alt="Image" /></td>
<td><img src="eluted-slide.png" alt="Image" /></td>
<td><img src="eluted-slide.png" alt="Image" /></td>
<td><img src="eluted-slide.png" alt="Image" /></td>
<td><img src="eluted-slide.png" alt="Image" /></td>
<td><img src="eluted-slide.png" alt="Image" /></td>
</tr>
<tr>
<td>Elution efficiency</td>
<td><strong>100%</strong></td>
<td><strong>100%</strong></td>
<td><strong>99%</strong></td>
<td><strong>99%</strong></td>
<td><strong>100%</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

The elution efficiency was over 99% for all 6 markers of the panel.

The method used with LabSat® shows efficient strip-off.
Elution study

**Impact on morphology**
The impact of repeated heating cycles was assessed for 3 markers.

Following 5 elution cycles on LabSat®, the results show no apparent degradation of the tissue morphology, including nuclear morphology, and epitope detection.

<table>
<thead>
<tr>
<th></th>
<th>1 AR</th>
<th>2 AR</th>
<th>3 AR</th>
<th>4 AR</th>
<th>5 AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1</td>
<td><img src="PD-L1_1.jpg" alt="Image" /></td>
<td><img src="PD-L1_2.jpg" alt="Image" /></td>
<td><img src="PD-L1_3.jpg" alt="Image" /></td>
<td><img src="PD-L1_4.jpg" alt="Image" /></td>
<td><img src="PD-L1_5.jpg" alt="Image" /></td>
</tr>
<tr>
<td>NI</td>
<td>2,1</td>
<td>2,6</td>
<td>2,9</td>
<td>3,6</td>
<td>3,8</td>
</tr>
<tr>
<td>CD8</td>
<td><img src="CD8_1.jpg" alt="Image" /></td>
<td><img src="CD8_2.jpg" alt="Image" /></td>
<td><img src="CD8_3.jpg" alt="Image" /></td>
<td><img src="CD8_4.jpg" alt="Image" /></td>
<td><img src="CD8_5.jpg" alt="Image" /></td>
</tr>
<tr>
<td>NI</td>
<td>6,5</td>
<td>9,1</td>
<td>9,4</td>
<td>9,2</td>
<td>9,0</td>
</tr>
<tr>
<td>PD-1</td>
<td><img src="PD-1_1.jpg" alt="Image" /></td>
<td><img src="PD-1_2.jpg" alt="Image" /></td>
<td><img src="PD-1_3.jpg" alt="Image" /></td>
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</tr>
<tr>
<td>NI</td>
<td>7,1</td>
<td>6,8</td>
<td>7,5</td>
<td>7,1</td>
<td>6,4</td>
</tr>
</tbody>
</table>
6-plex workflow

- **Tissue deparaffinization**
- **Reagents / buffers loading on device**

### Antigen retrieval
- Blocks
- AbI
- AbII - HRP

### Amplification (TSA)

### Elution

**Intra-slide procedure:** 2 min

**Slide stays in the machine**

**Reagent swap**

- **Antigen retrieval**
- Blocks
- AbII - HRP

- **Amplification (TSA)**

- **Elution**

**Slide stays in the machine**

**Mounting & coverslipping**

**Microscopy**

+ **DAPI**
Multiplexing: results

Semi automated 6-plex with LabSat® and Opal®

4h12m

FFPE tonsil, 6-plex + DAPI staining in 4h12 with LabSat® and Opal® kit

- FoxP3
- PD-L1
- PD-1
- CD68
- CD8
- CK
- DAPI
Multiplexing: results

- High signal-to-background ratio for each marker
- Signal uniformity (no gradient) across tissue section
- Efficient antibody elution: over 99% for all 6 markers
- Tissue morphology and epitopes are highly preserved
- Reproducible results with high degree of consistency
Protocol transfer
Protocol transfer method

*Is this tonsil-optimized protocol applicable to my cancer tissue samples?*

**Transfer strategy:**
- Apply tonsil-optimized protocol
- Evaluate performance and define which markers need re-optimization
- Modify protocol parameters to reach desired performance

**Case study:** Non small-cell lung carcinoma (NSCLC) whole-tumor and TMA sections

**Objective:** Reach acceptable performance on NSCLC in a minimum number of optimization steps
Protocol parameters

LabSat® offers flexibility for optimization:

• Modify antigen retrieval temperature
• Modify incubation times
• Additional steps (blocking, etc.)
• Reagents titrations
Direct application of the protocol on NSCLC showed lower intensity levels than expected for all markers compared to tonsil. **Re-optimization needed.**
6-plex TSA transfer to NSCLC whole-tumor

Protocol adjustments:
- Antigen retrieval temperature increase
- Opal 540 (CD8) concentration increase

<table>
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<th>CK</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routine IHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&quot;Tonsil-optimized&quot; protocol</td>
<td></td>
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<td></td>
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<tr>
<td>Adjusted for NSCLC</td>
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</tbody>
</table>

Conclusion:
Optimized TSA-based 6-plex protocol resulted in appropriate detection of all markers on NSCLC
Transfer the optimized 6-plex for NSCLC from FFPE whole-tumor to TMA.

**Conclusion:**
Optimized TSA 6-plex protocol showed appropriate detection of all markers on a NSCLC TMA comparably to standard IHC.
6-plex TSA transfer to NSCLC TMA

Transfer the optimized 6-plex for NSCLC from FFPE whole-tumor to TMA.

Conclusion:
Various patterns of expression can be detected comparably to standard IHC

6-plex: 4h12m
Conclusions

Optimized Opal® panel on Labsat®
- Full 6-plex / 7-color assay on tonsil tissue in 4h12min
- Quality results (signal, uniformity, strip-off, morphology)

Protocol transfer to a different tissue type
- 6-plex / 7-color assay transferred to lung (same duration: 4h12min)
- Easy protocol adaptation (2-steps transfer)
Thank you for joining us.

Please send your questions to: communications@lunaphore.com