Katerina Mrazova

Staining Strategies of Biological Samples Prepared for Volume Microscopy
Summary

• On section vs. En bloc staining
• Staining reagents
  → osmium tetroxide
  → thiokarbohydrazide
  → uranyl acetate
  → lead aspartate
• Mostly used methods
  → OTO
  → rOTO
  → Hua
• Possible problems
• Alternative staining methods
Staining strategies
Staining reagents

- Osmium tetroxide
  - yellow crystals, highly oxidizing, volatile vapours
  - reaction with organic compounds (unsaturated bonds of fatty acids)
  - secondary fixation agent (membranes) as well as a contrasting agent
  - reduced osmium (+ $K_3[\text{Fe(CN)}_6]$ / $K_4[\text{Fe(CN)}_6]$)

- Thiokarbohydrazide
  - white to pale grey crystals, toxic, light-sensitive
  - very slightly soluble (0.5g/100g, 25°C)
  - attachment to osmium bound in the tissue enabling second osmium binding
Staining reagents

- Uranyl acetate
  - negative staining, on-section staining, en bloc staining since the 1960s
  - highly toxic, mildly radioactive
  - mostly reacts with nucleic acids and proteins
  - subject to rising legal restrictions
  - lanthanoids as a possible substitute

- Lead aspartate (Walton PbAsp, 1979)
  - aspartic acid + lead nitrate
  - lesser contaminations, lower pH than lead citrate
  - toxic, challenging preparation
## Conventional staining strategies

<table>
<thead>
<tr>
<th>Incubation steps</th>
<th>r-OTO</th>
<th>OTO</th>
<th>Hsu</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.</strong></td>
<td>2% OsO₄, 2.5% ferricyanide, 0.15 M CaCl₂, pH 7.4</td>
<td>2% OsO₄, unbuffered</td>
<td>2% OsO₄, 0.15 M CaCl₂, pH 7.4</td>
</tr>
<tr>
<td></td>
<td>1.5 h @ rt</td>
<td>1.5 h @ rt</td>
<td>1.5 h @ rt</td>
</tr>
<tr>
<td></td>
<td>No wash</td>
<td>No wash</td>
<td>No wash</td>
</tr>
<tr>
<td><strong>2.</strong></td>
<td></td>
<td></td>
<td>2.5% ferricyanide, 0.15 M CaCl₂, pH 7.4</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1.5 h @ rt</td>
</tr>
<tr>
<td><strong>3.</strong></td>
<td>1% TCH, unbuffered</td>
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<td>1% TCH, unbuffered</td>
</tr>
<tr>
<td></td>
<td>0.75 h @ 50 °C</td>
<td>0.75 h @ rt</td>
<td>0.75 h @ 40 °C</td>
</tr>
<tr>
<td></td>
<td>0.5 h wash in water x 2</td>
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<td>0.5 h wash in water x 2</td>
</tr>
<tr>
<td><strong>4.</strong></td>
<td>2% OsO₄, unbuffered</td>
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</tr>
<tr>
<td></td>
<td>1.5 h @ rt</td>
<td>1.5 h @ rt</td>
<td>1.5 h @ rt</td>
</tr>
<tr>
<td></td>
<td>0.5 h wash in water x 2</td>
<td>0.5 h wash in water x 2</td>
<td>0.5 h wash in water x 2</td>
</tr>
<tr>
<td><strong>5.</strong></td>
<td>1% uranyl acetate, unbuffered</td>
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<td>1% uranyl acetate, unbuffered</td>
</tr>
<tr>
<td></td>
<td>2 h @ 50 °C</td>
<td>2 h @ 50 °C</td>
<td>overnight @ 4 °C, 2 h @ 50 °C</td>
</tr>
<tr>
<td></td>
<td>0.5 h wash in water x 2</td>
<td>0.5 h wash in water x 2</td>
<td>0.5 h wash in water x 2</td>
</tr>
<tr>
<td><strong>6.</strong></td>
<td>Lead aspartate, pH 5.0</td>
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</tr>
<tr>
<td></td>
<td>2 h @ 50 °C</td>
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<tr>
<td></td>
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</tr>
</tbody>
</table>

dehydration, infiltration and embedding
OTO, Seligman, 1966

- Firstly published to enhance the contrast of osmicated sections on grids
- Variations of the protocol used for en bloc staining
- Procedure
  - glutaraldehyde (2.5% in buffer, RT/4 °C, 4h)
  - washing buffer (3x15min)
  - OsO₄ (2% in buffer, RT, 1.5h)
  - washing buffer (3x15min)
  - thiocarbohydrazide (1% in water, 50°C, 1h)
  - washing water (3x15min)
  - OsO₄ (1% in water, RT, 1h)
  - washing water (3x15min)
  - uranyl acetate (1% in water, 50°C, 2h)
  - washing water (3x15min)
  - Walton lead aspartate (50°C, 2h)
  - washing water (3x15min)
  - acetone (30% » 50% » 70% » 80% » 90% » 95% » 100%, RT, 15min)
  - epon (in acetone, 1:2 » 1:1 » 2:1 » 2x pure resin, RT, 1h, last overnight, curing 60°C 48h)
rOTO, Willingham, 1983

- Improvement of fixation/staining of lipidic structures and membranes before EtOH dehydration

- Procedure
  - glutaraldehyde (2.5% in buffer, RT/4 °C, 4h)
  - washing buffer (3x15min)
  - OsO₄ + K₄[Fe(CN)]₆ (2%+2.5% in buffer, RT, 1.5h)
  - washing buffer (3x15min)
  - thiocarbohydrazide (1% in water, 50°C, 1h)
  - washing water (3x15min)
  - OsO₄ (1% in water, RT, 1h)
  - washing water (3x15min)
  - uranyl acetate (1% in water, 50°C, 2h)
  - washing water (3x15min)
  - Walton lead aspartate (50°C, 2h)
  - washing water (3x15min)
  - acetone (30% » 50% » 70% » 80% » 90% » 95% » 100%, RT, 15min)
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rOTO, Willingham, 1983

- Improvement of fixation/staining of lipidic structures and membranes before EtOH dehydration
- Procedure
  → glutaraldehyde (2.5% in buffer RT/4°C 4h)
  → washing buffer
  → OsO₄ + K₄[Fe(CN)₆]
  → washing buffer
  → thiocarbohydride
  → washing water
  → OsO₄ (1% in water)
  → washing water
  → uranyl acetate
  → washing water
  → Walton lead acetate
  → washing water
  → acetone (30%)
  → Epon (in acetone, 1:2 > 1:1 > 2:1 > ≥x pure resin, 1 h, 1st overnight, curing 60°C 48h)
Hua, 2015

- Main changes in Os and U steps to achieve high-contrast staining throughout large tissue blocks

- Procedure
  - $2.5\%$ glutaraldehyde (in buffer, RT/4 °C, 4h)
  - washing buffer (3x15min)
  - $\text{OsO}_4$ (2% in buffer, RT, 1.5h)
  - $K_4[\text{Fe(CN)}_6]$ (2.5% in buffer, RT, 1.5h)
  - thiocarbohydrazide (1% in water, 40°C, 45min)
  - washing water (3x15min)
  - $\text{OsO}_4$ (2% in water, RT, 1.5h)
  - washing water (3x15min)
  - uranyl acetate (1% in water, 4°C overnight, 50°C 2h)
  - washing water (3x15min)
  - Walton lead aspartate (50°C, 2h)
  - washing water (3x15min)
  - acetone (30% » 50% » 70% » 80% » 90% » 95% » 100%, RT, 15min)
  - epon (in acetone, 1:2 » 1:1 » 2:1 » 2x pure resin, RT, 1h, last overnight, curing 60°C 48h)
Hua, 2015

- Main changes in Os and U steps to achieve high-contrast staining throughout large tissue blocks

- Procedure:

  1. OsO₄ staining
  2. Washing
  3. Reduced Os staining
  4. After wash

→ acetone (30% → 50% → 70% → 80% → 90% → 95% → 100%, RT, 15min)
→ epon (in acetone, 1:2 → 1:1 → 2:1 → 2x pure resin, RT, 1h, last overnight, curing 60°C 48h)
Main changes in Os and U steps to achieve high-contrast staining throughout large tissue blocks

→ acetone (30% » 50% » 70% » 80% » 90% » 95% » 100%, RT, 15min)
→ epon (in acetone, 1:2 » 1:1 » 2:1 » 2x pure resin, RT, 1h, last overnight, curing 60°C 48h)
Issues
Issues
Alternative strategies - lanthanoids

• Kuipers, 2020
  • Use of neodymium acetate as uranyl substitute
  • Similar chemical properties due to the position in the table of elements therefore assumption → very similar in binding to tissue
  • Procedure
    → standard fixation and post-fixation by osmium
    → 4% NdAc 30 / 60 / 120 min at RT
    → dehydration and resin embedding
Alternative strategies - lanthanoids
Alternative strategies - lanthanoids

- Pinto, 2021
  - Testing commercially available uranyl-less staining agents on cilia
  - UA-zero (Agar Scientific) → ytterbium chloride + phosphothungstid acid
  - UAR (EMS) → samarium and gadolinium triacetate
  - Procedure
    → glutaraldehyde (2.5% in buffer, 4°C, overnight)
    → wash (buffer)
    → OsO₄ (1% in water, RT, 1h)
    → wash (water)
    → UA/Ua zero/UAR/no stain (1% in water 30min / no dilution 30min / 1:4 in water 30min / no stain)
    → ethanol (50% ➔ 70% ➔ 90% ➔ 100%)
    → propylene oxide + resin
Alternative strategies - lanthanoids

- Pinto, 2021

<table>
<thead>
<tr>
<th>Grid</th>
<th>No stain</th>
<th>UA</th>
<th>UAR</th>
<th>UAZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>En bloc</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Procedure:
- Test incubation time
- UA-zen
- UAR (EMS)
- Procedural variation

Ytterbium chloride + phosphothungstic acid → samarium and gadolinium triacetate
• Pinto, 2
• Testing com
• UA-zero (Ag
• UAR (EMS) –
• Procedure
  → glutat
  → wash
  → OsO₄ (  
  → wash
  → UA/U₃ (  
  → ethan
  → propy

(Planter 30min / no stain)
Alternative strategies - lanthanoids

• Moscardini, 2020

• Use of ytterbium chloride and phosphotungstic acid (PTA) as an alternative stain
• Commercially available as UA zero (Agar Scientific)
• For negative staining, on-section staining, en bloc staining
• Ytterbium high electron scattering power, PTA previously proven to enhance Uac staining
Alternative strategies - Lanthanoids

- Moscardini,
- Use of ytterbium does not interfere with the sample's ultrastructure
- Commercially available
- For negative staining
- Ytterbium high electrical conductivity as compared to Uac staining
Alternative strategies - Lanthanoids

• Moscardini, 2020
  • Use of ytterbium chloride and phosphotungstic acid as an alternative stain
  • Commercially available as UA zero (Agar Scientific)
  • For negative staining, on-section staining, en bloc staining
  • Ytterbium high electron scattering power, PTA previously proven to enhance Uac staining
  • Procedure
    → glutaraldehyde (2% in buffer, 4°C, overnight)
    → OsO₄ + K₃[Fe(CN)₆] (1% +1% in buffer)
    → washing
    → optimized X Solution (ratio 15 YbCl₃ : 1 PTA), PTA 3.2 mM, YbCl₃ 48 mM alone and UA 3% (1h)
    → dehydration, resin embedding
Alternative strategies - lanthanoids

- Moscardini, 2020

- Use of ytterbium
- Commercial
- For negative
- Ytterbium
- Procedure
  → glut
  → OsO₄
  → wash
  → opti
  → dehy

UA 3%
X SOL
MES 20 mM

% (1h)
Alternative strategies - Lanthanoids

- Moscovite
- Use of ytterbium
- Commercial products
- For negative stain
- Ytterbium
- Procedures:
  - gl
  - Osmium
  - wash
  - open
  - de
Thank you for your attention.