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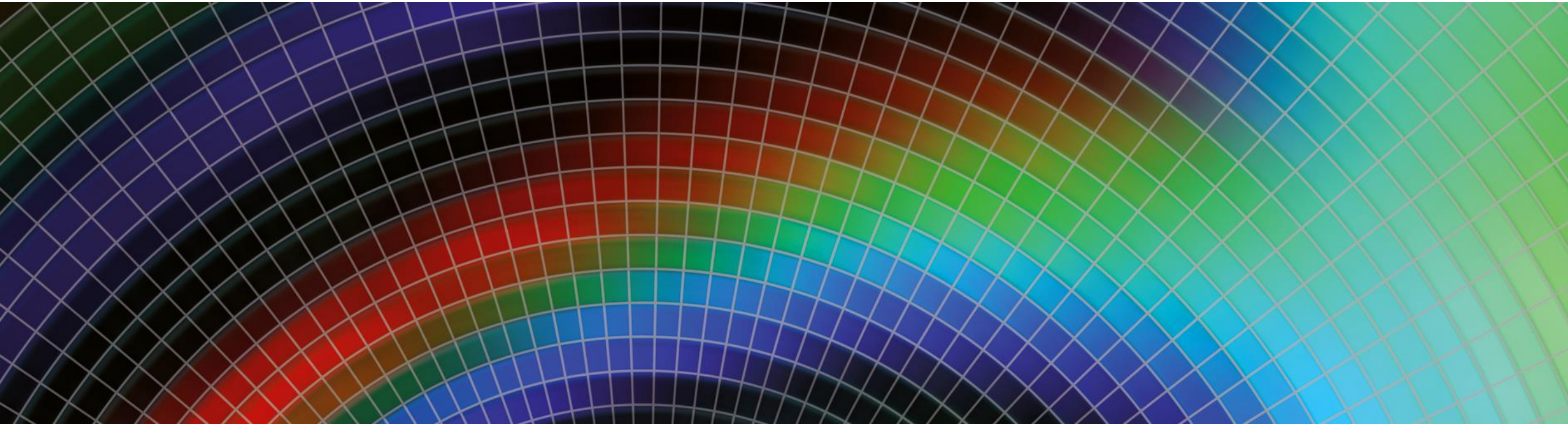


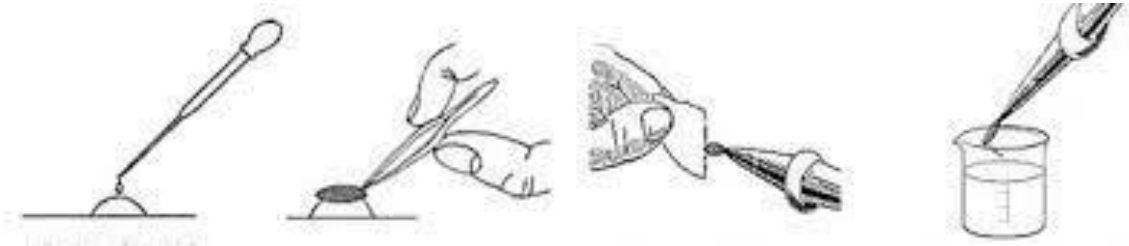
Katerina Mrazova

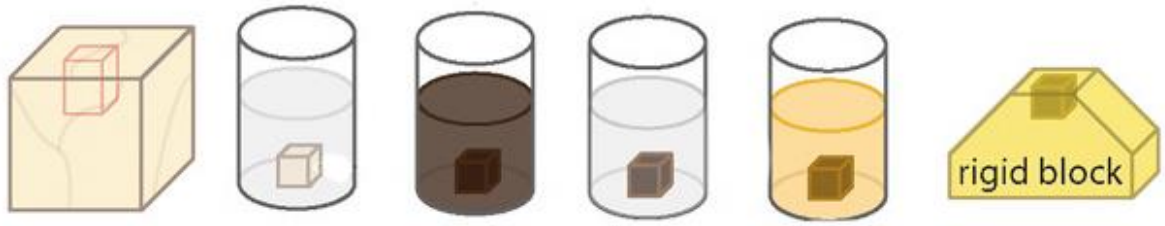
Staining Strategies of Biological Samples Prepared for Volume Microscopy

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

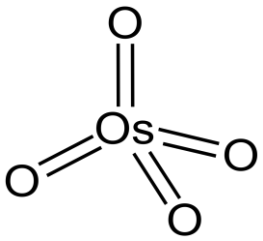
Staining strategies



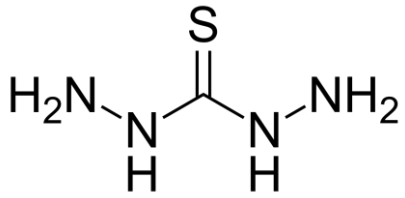


Staining reagents

- 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 (+ $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$)



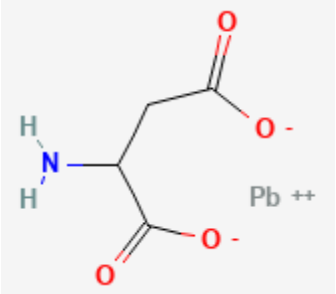
- Thiokarbohydrazide
  → white to pale grey crystals, toxic, light-sensitive
  → very slightly soluble (0.5g/100g, 25°C)
  → attachment to osmium bound in the tissue enabling second osmium binding



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

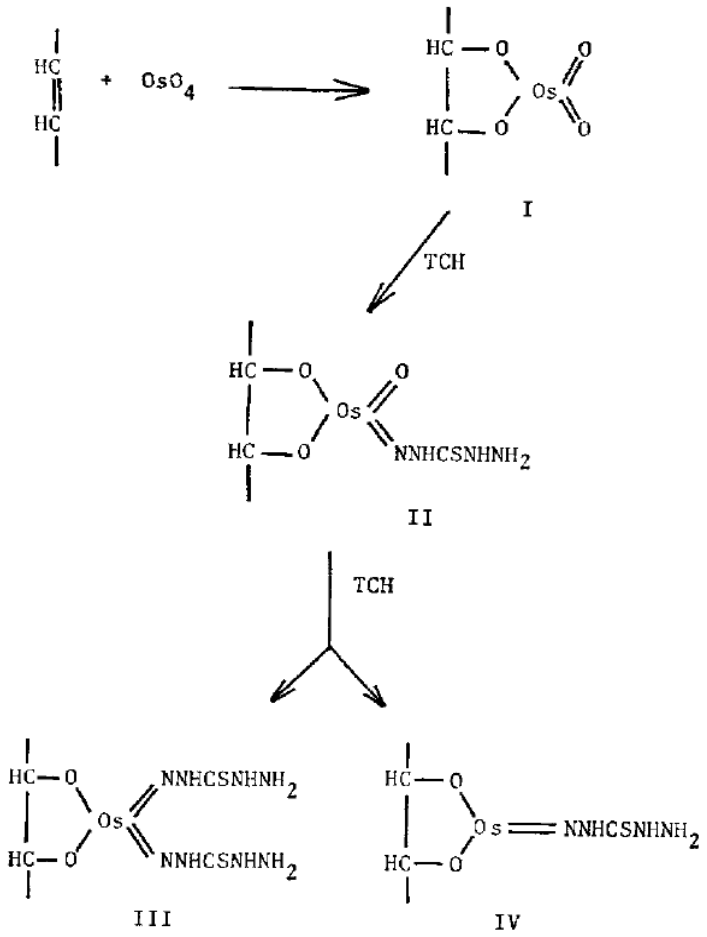


Conventional staining strategies

	protocol		
incubation steps	r OTO	OTO	Hua
1.	2% OsO4, 2.5% ferrocyanide, 0.15 M Cac, pH 7.4	2% OsO4, unbuffered	2% OsO4, 0.15 M Cac, pH 7.4
	1.5 h @ rt	1.5 h @ rt	1.5 h @ rt
	No wash		
2			2.5% ferrocyanide, 0.15 M Cac, pH 7.4
			1.5 h @ rt
	0.5 h wash in water x 2		
3	1% TCH, unbuffered	1% TCH, unbuffered	1% TCH, unbuffered
	0.75 h @ 50 ºC	0.75 h @ rt	0.75 h @ 40 ºC
	0.5 h wash in water x 2		
4	2% OsO4, unbuffered 1.5 h @ rt	2% OsO4, unbuffered 1.5 h @ rt	2% OsO4, unbuffered 1.5 h @ rt
	0.5 h wash in water x 2		
5	1 % uranyl acetate, unbuffered	1 % uranyl acetate, unbuffered	1% uranyl acetate, unbuffered
	2 h @ 50 ºC	2 h @ 50 ºC	overnight @ 4 ºC, 2 h @ 50 ºC
	0.5 h wash in water x 2		
6	Lead aspartate, pH 5.0	Lead aspartate, pH 5.0	Lead aspartate, pH 5.0
	2 h @ 50 ºC	2 h @ 50 ºC	2 h @ 50 ºC
	0.5 h wash in water x 2		
	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)
  - → **OsO_4 (2% in buffer, RT, 1,5h)**
  - → washing buffer (3x15min)
  - → **thiocarbohydrazide (1% in water, 50°C, 1h)**
  - → washing water (3x15min)
  - → **OsO_4 (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)
  - → **OsO_4 + $K_4[Fe(CN)_6]$ (2%+2,5% in buffer, RT, 1,5h)**
  - → washing buffer (3x15min)
  - → **thiocarbohydrazide (1% in water, 50°C, 1h)**
  - → washing water (3x15min)
  - → **OsO_4 (1% in water, RT, 1h)**
  - → washing water (3x15min)
  - → uranyl acetate (1% in water, 50°C, 2h)
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  - → **OsO_4 (1% in**
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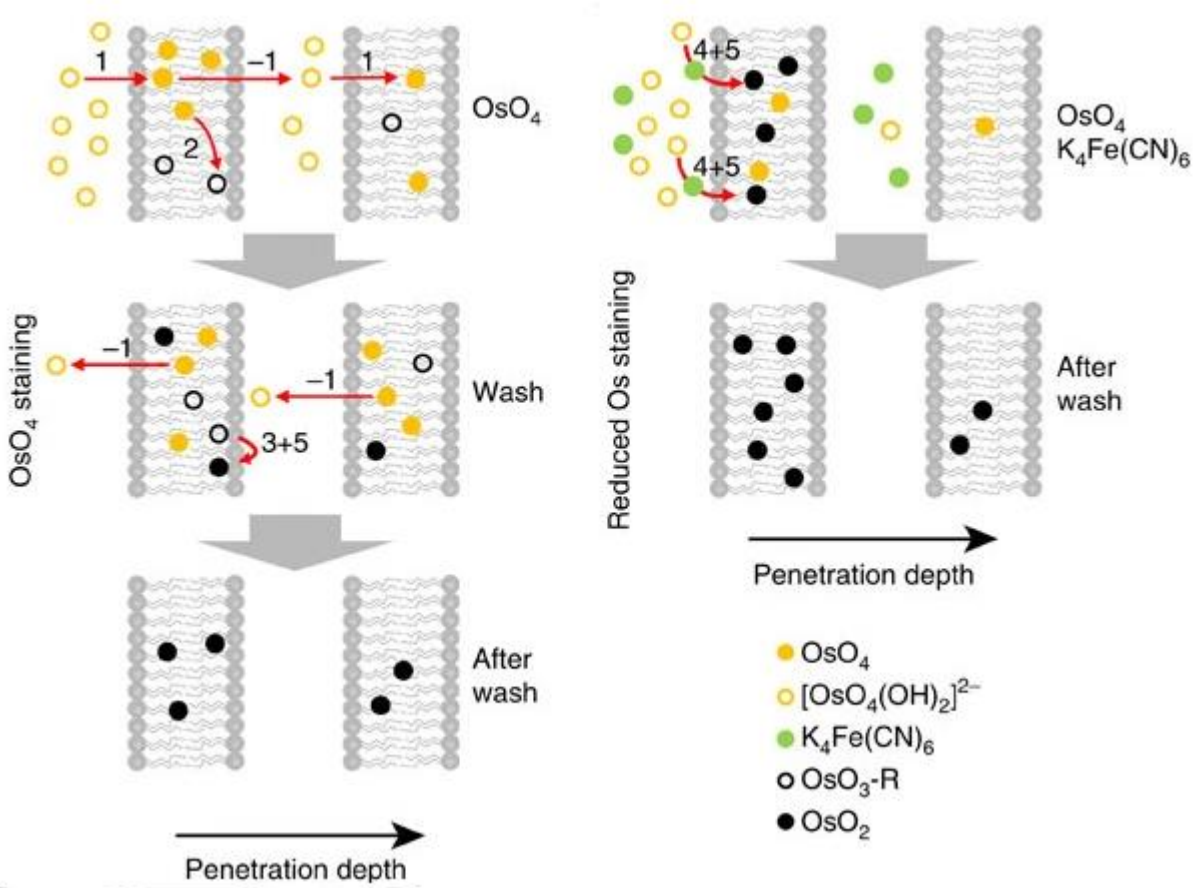

Hua, 2015

- 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)
  - → **OsO_4 (2% in buffer, RT, 1,5h)**
  - → **$K_4[Fe(CN)_6]$ (2,5% in buffer, RT, 1,5h)**
  - → **thiocarbohydrazide (1% in water, 40°C, 45min)**
  - → washing water (3x15min)
  - → **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)
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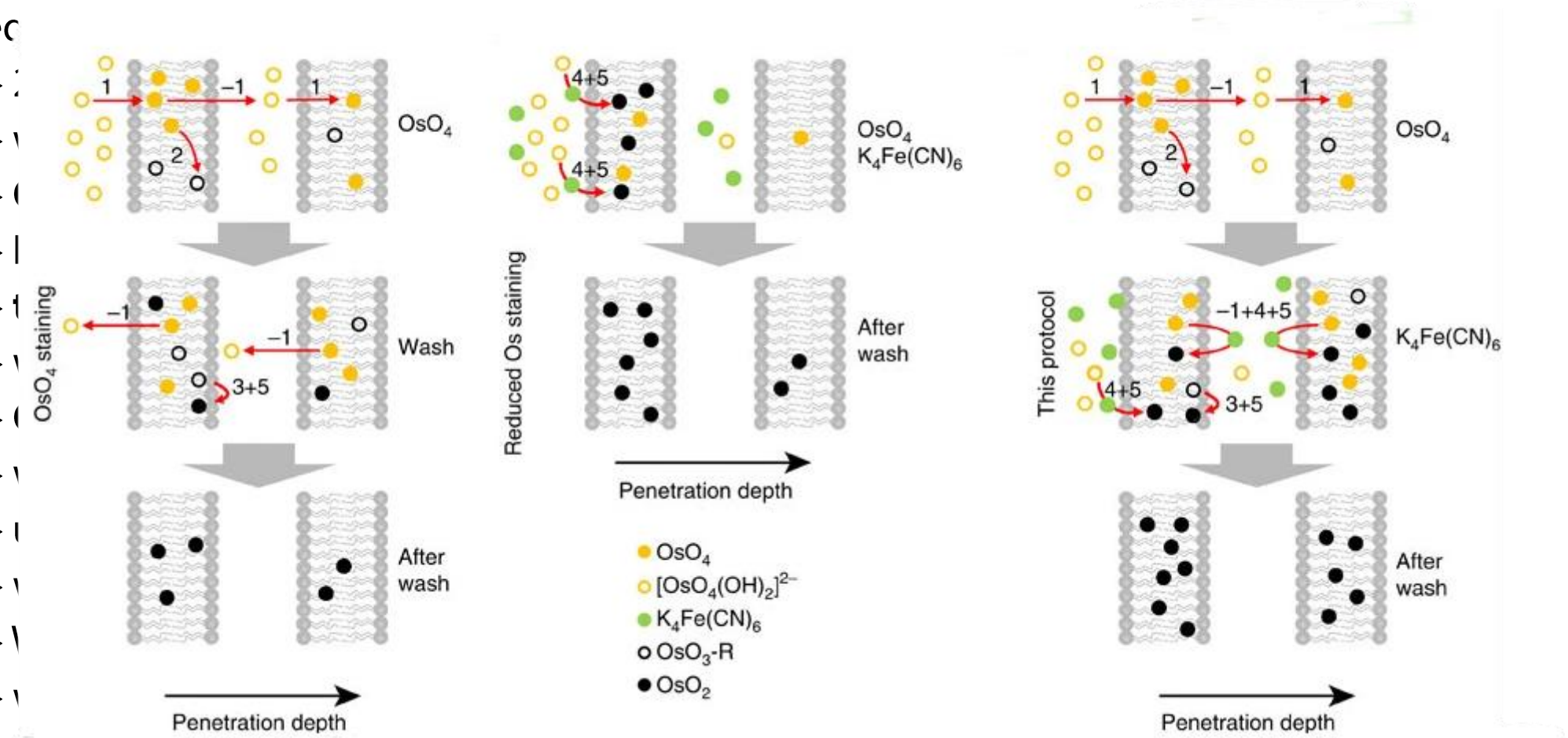

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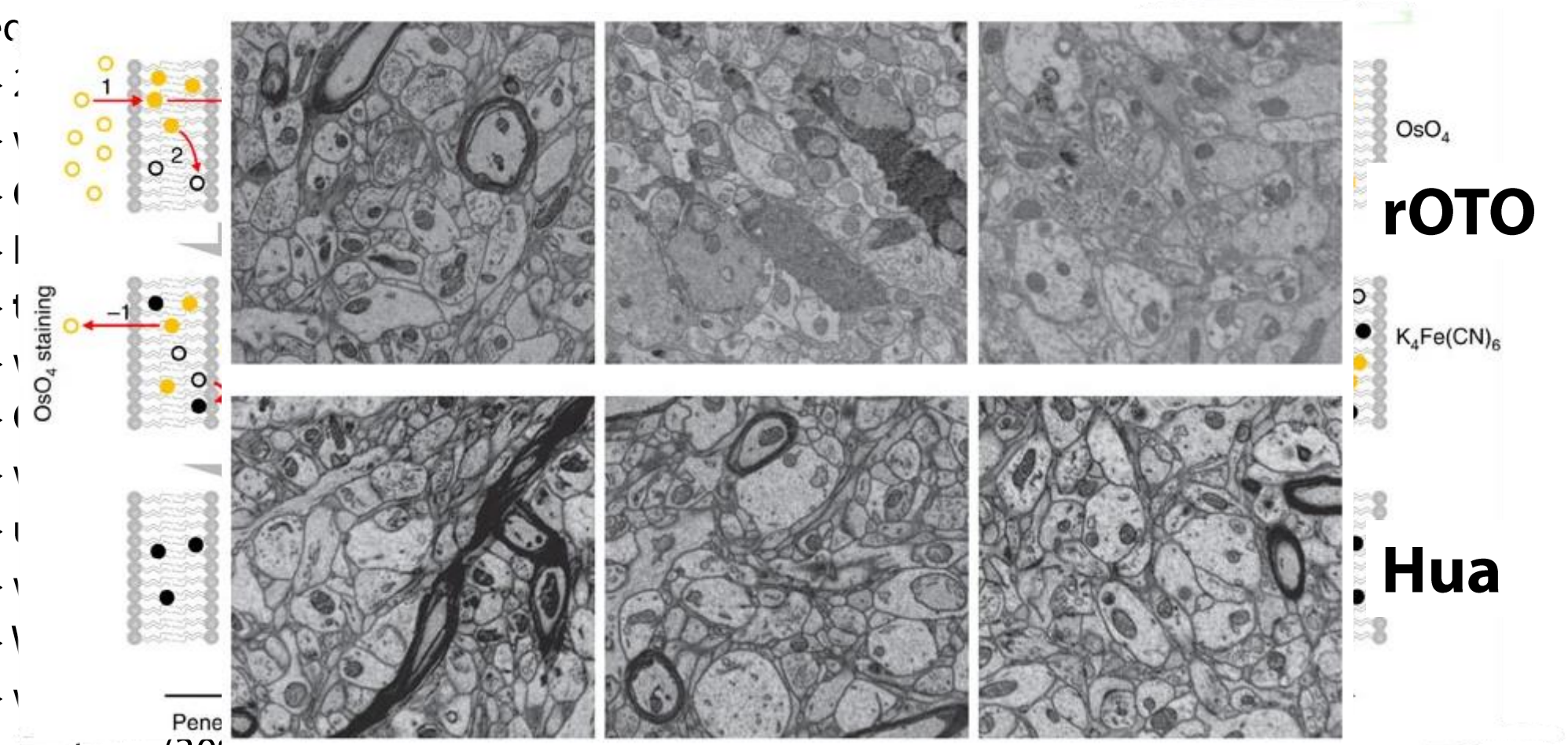

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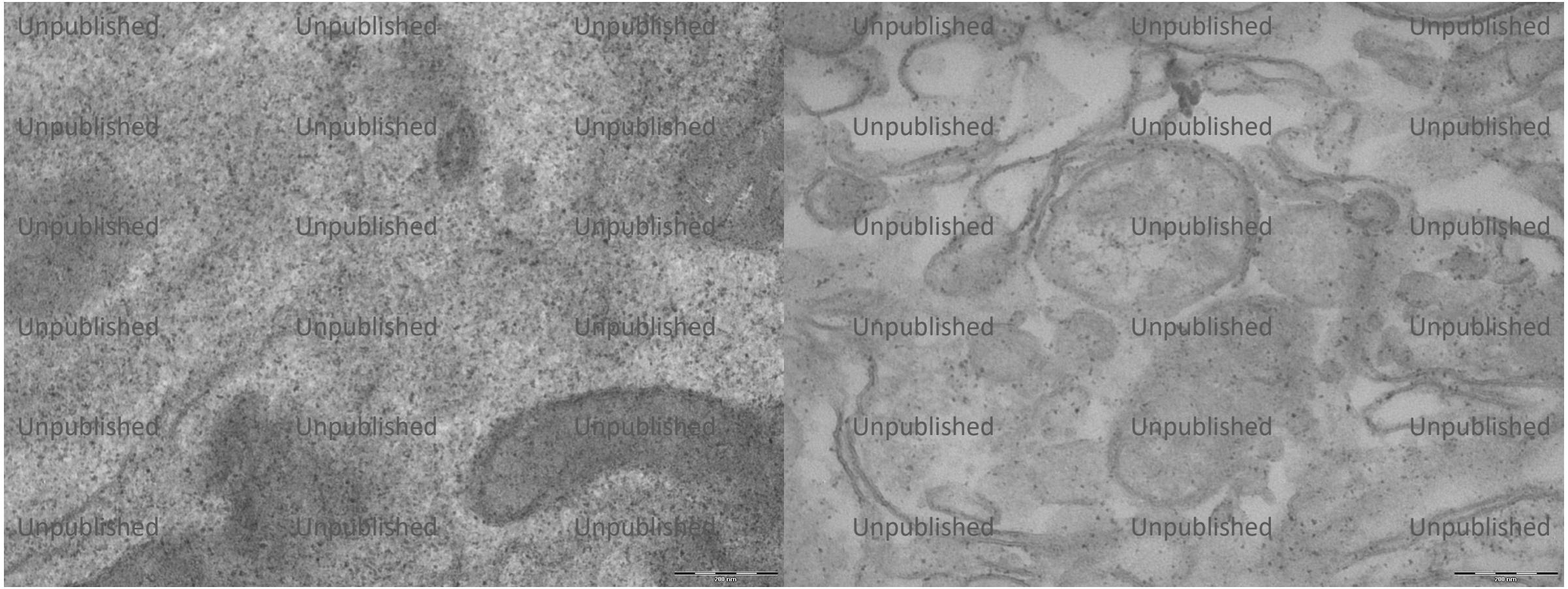
Issues



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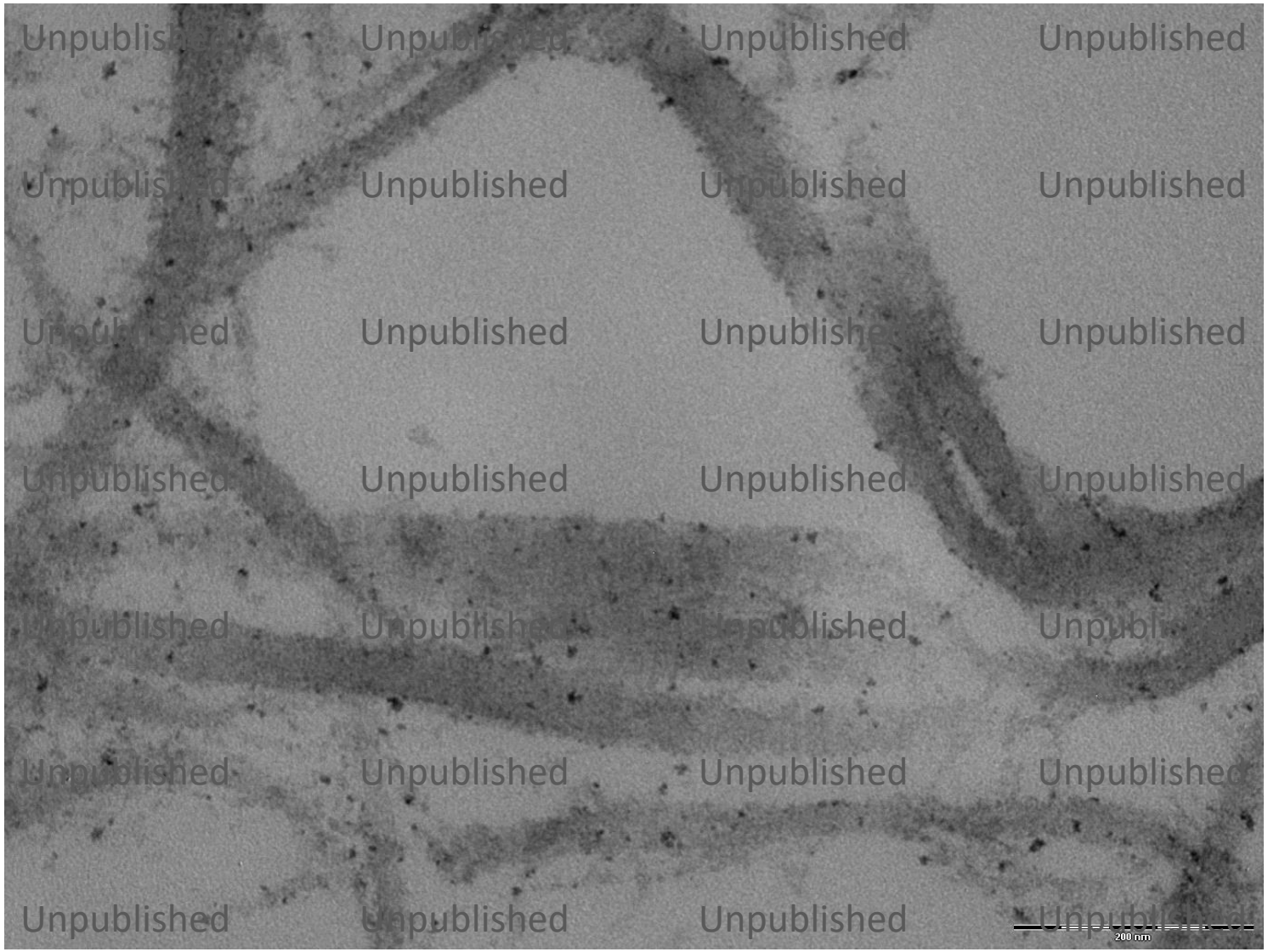
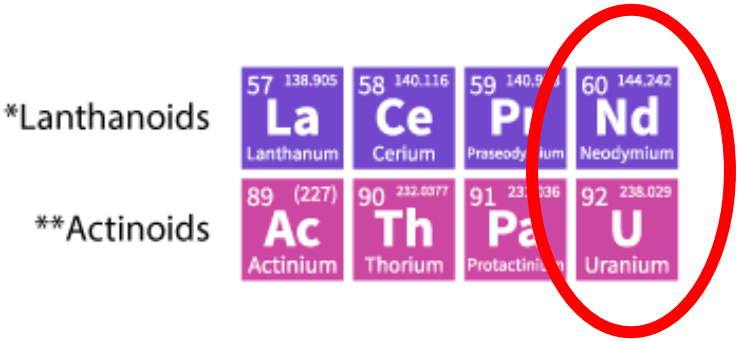


Alternative strategies - lanthanoids

- Kuipers, 2020

- Use of neodymium acetate as uranyl substitute
- Simillar chemical properties due to the position in the table of elements therefore assumption → very similar in binding to tissue
- Procedure
  - → standard fixation and postfixation by osmium
  - → 4% NdAc 30 / 60 / 120 min at RT
  - → dehydration and resin embedding



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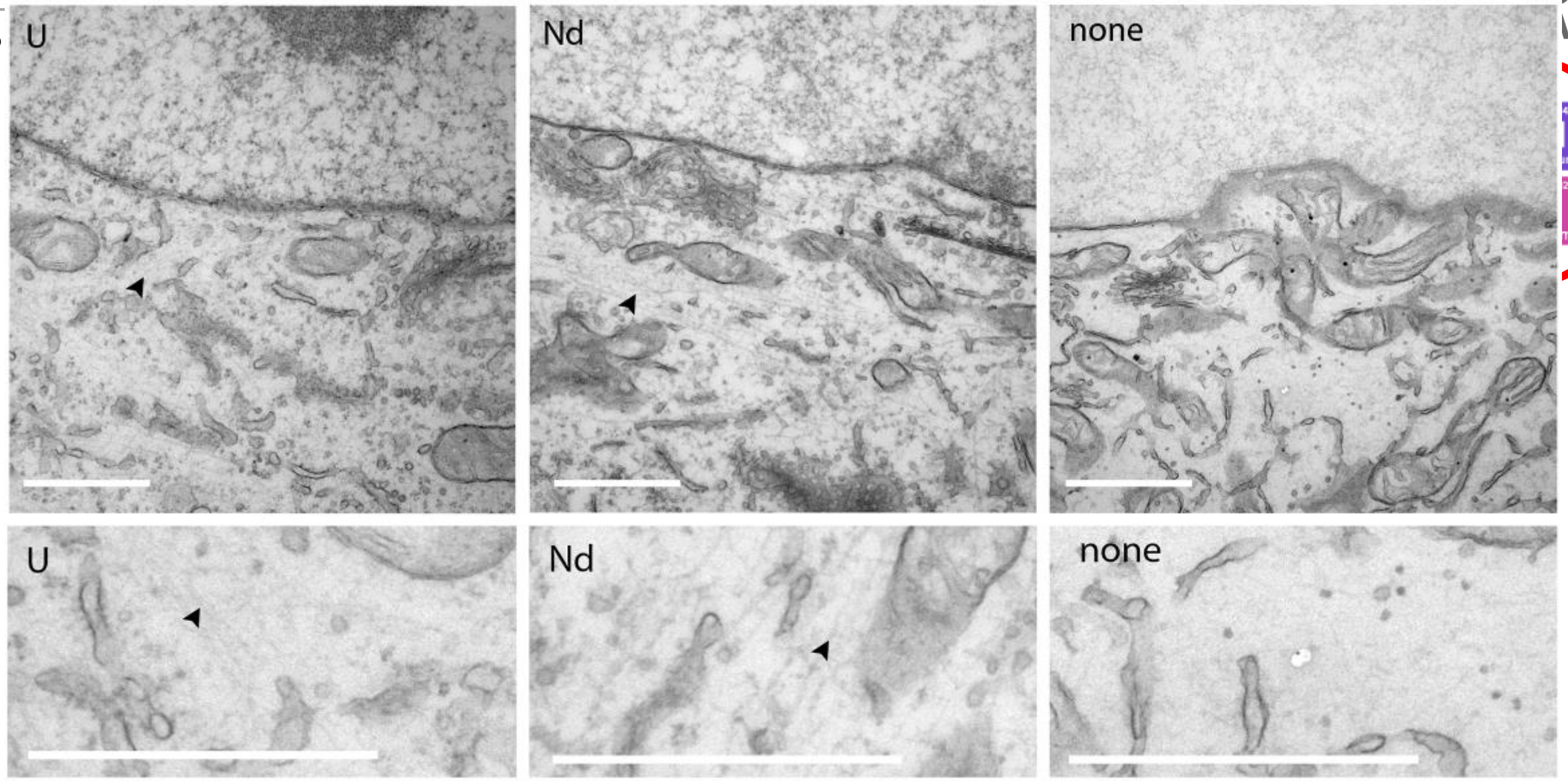

Alternative strategies - lanthanoids

- 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)
  - → OsO_4 (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
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- UA-ze
- UAR (E
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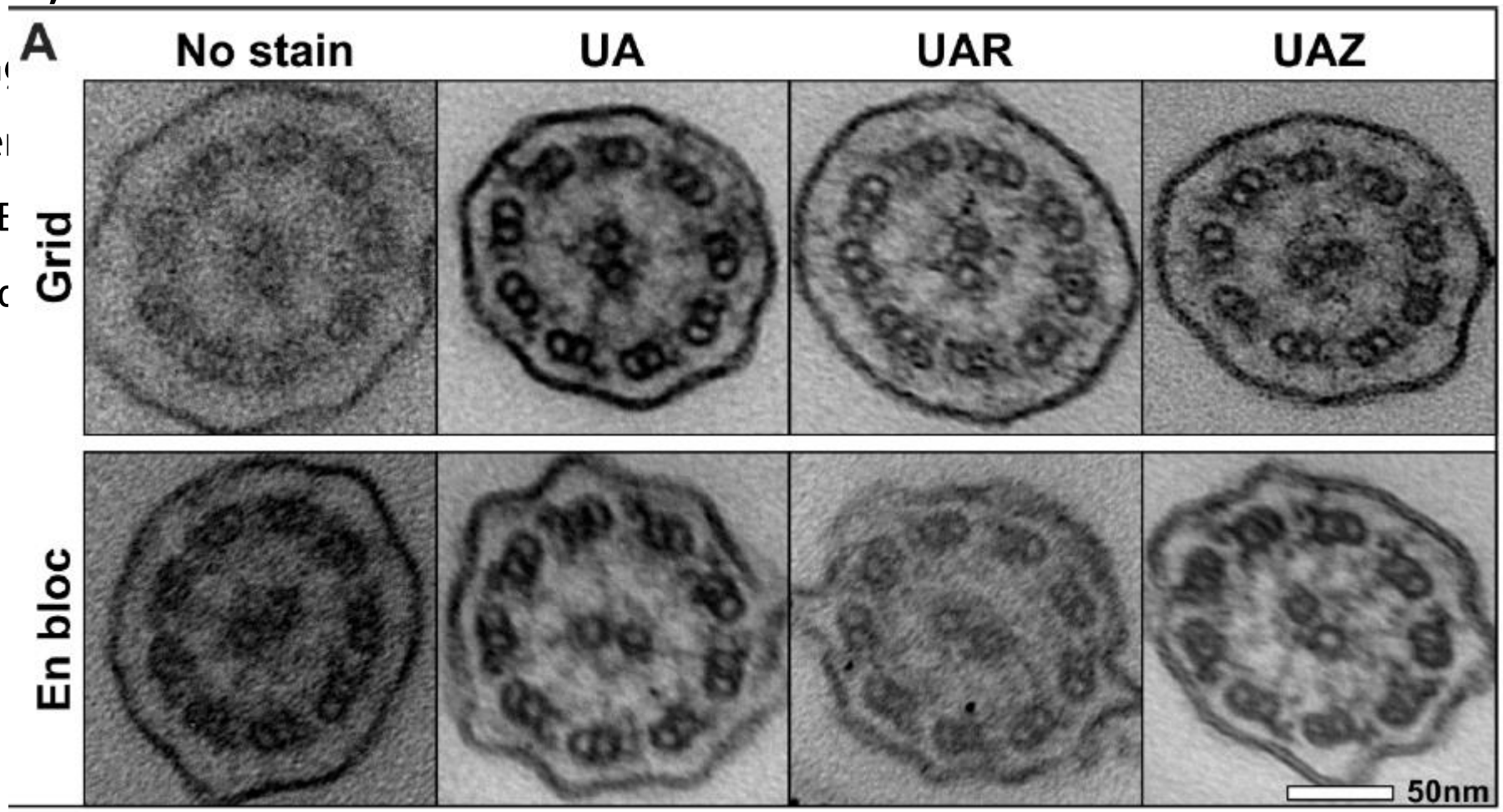


)min / no stain)

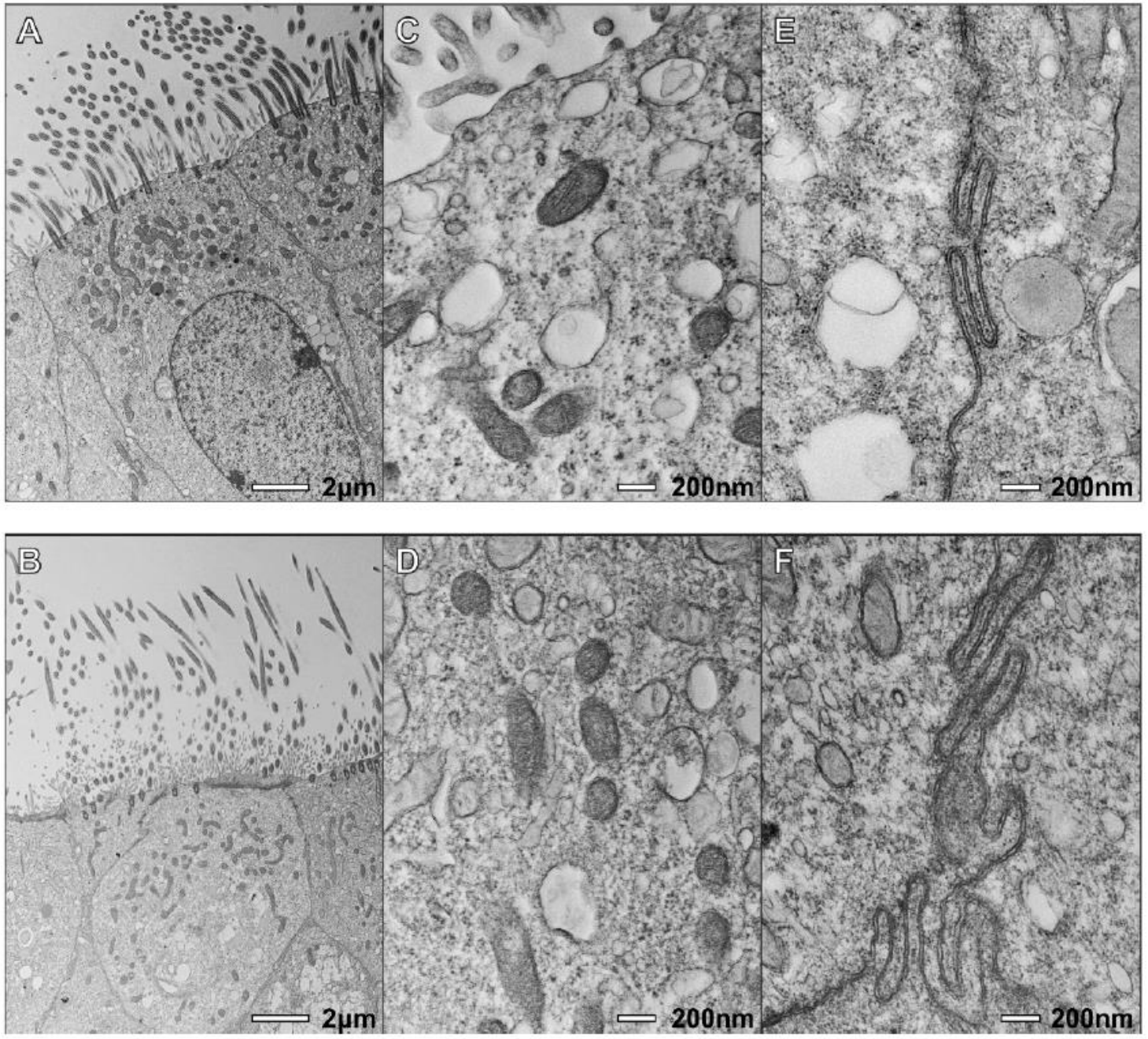
Alterna

- Pinto, 2

- Testing com
- UA-zero (Ag
- UAR (EMS) –
- Procedure
  - → gluta
  - → wash
  - → OsO_4 (
  - → wash
  - → UA/U
  - → ethan
  - → propy

UA en bloc

UAZ en bloc



ater 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

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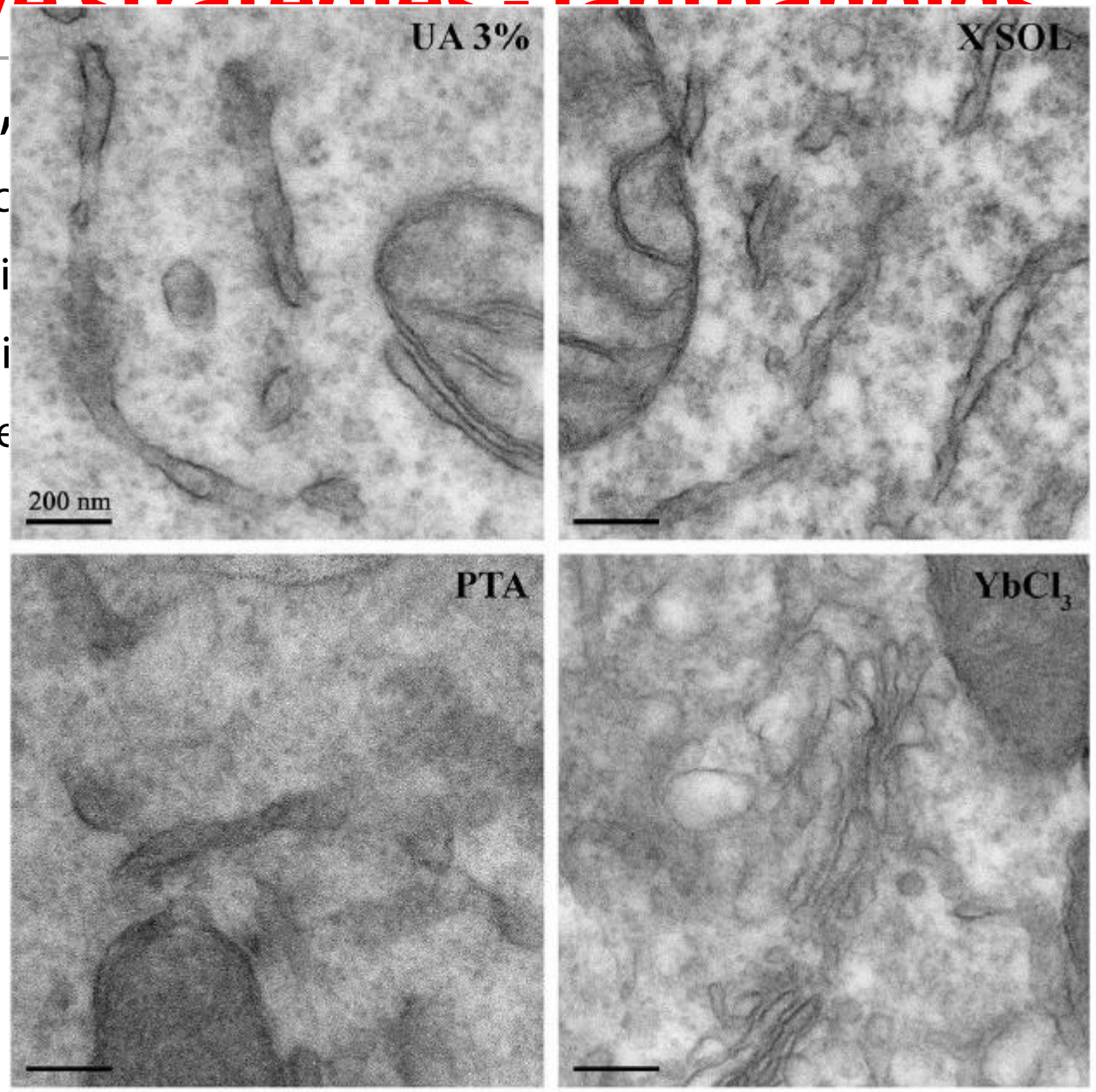
- Use of ytterbium c
- Commercially avai
- For negative staini
- Ytterbium high ele :e Uac staining



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)
  - → OsO_4 + $K_3[Fe(CN)_6]$ (1% +1% in buffer)
  - → washing
  - → optimized X Solution (ratio 15 YbCl : 1 PTA), PTA 3.2 mM, $YbCl_3$ 48 mM alone and UA 3% (1h)
  - → dehydration, resin embedding

Alternative strategies - lanthanoids

- Moscardini, 2020
  - Use of ytte
  - Commerci
  - For negati
  - Ytterbium
  - Procedure
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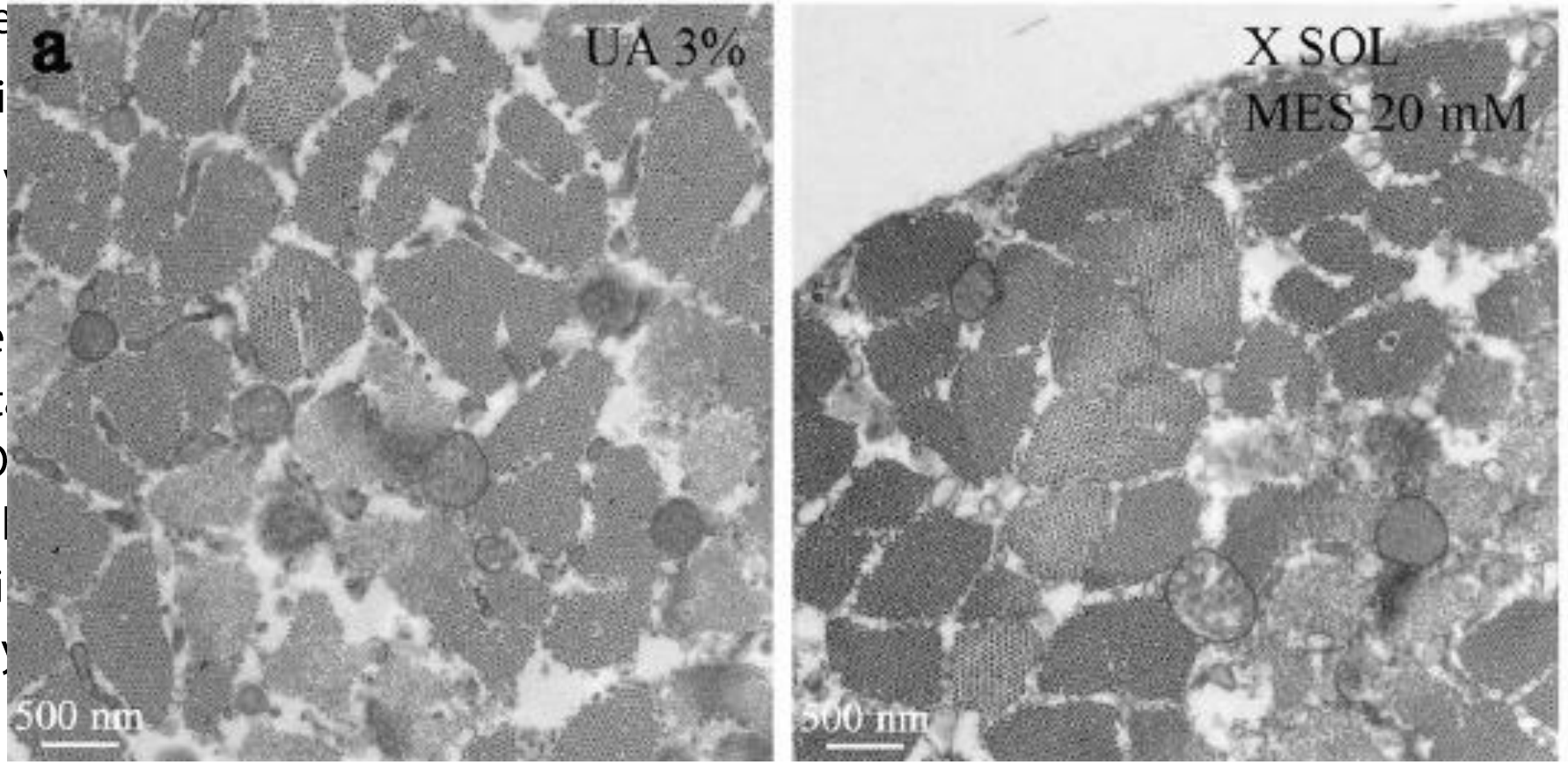

% (1h)

Alternative strategies - lanthanoids

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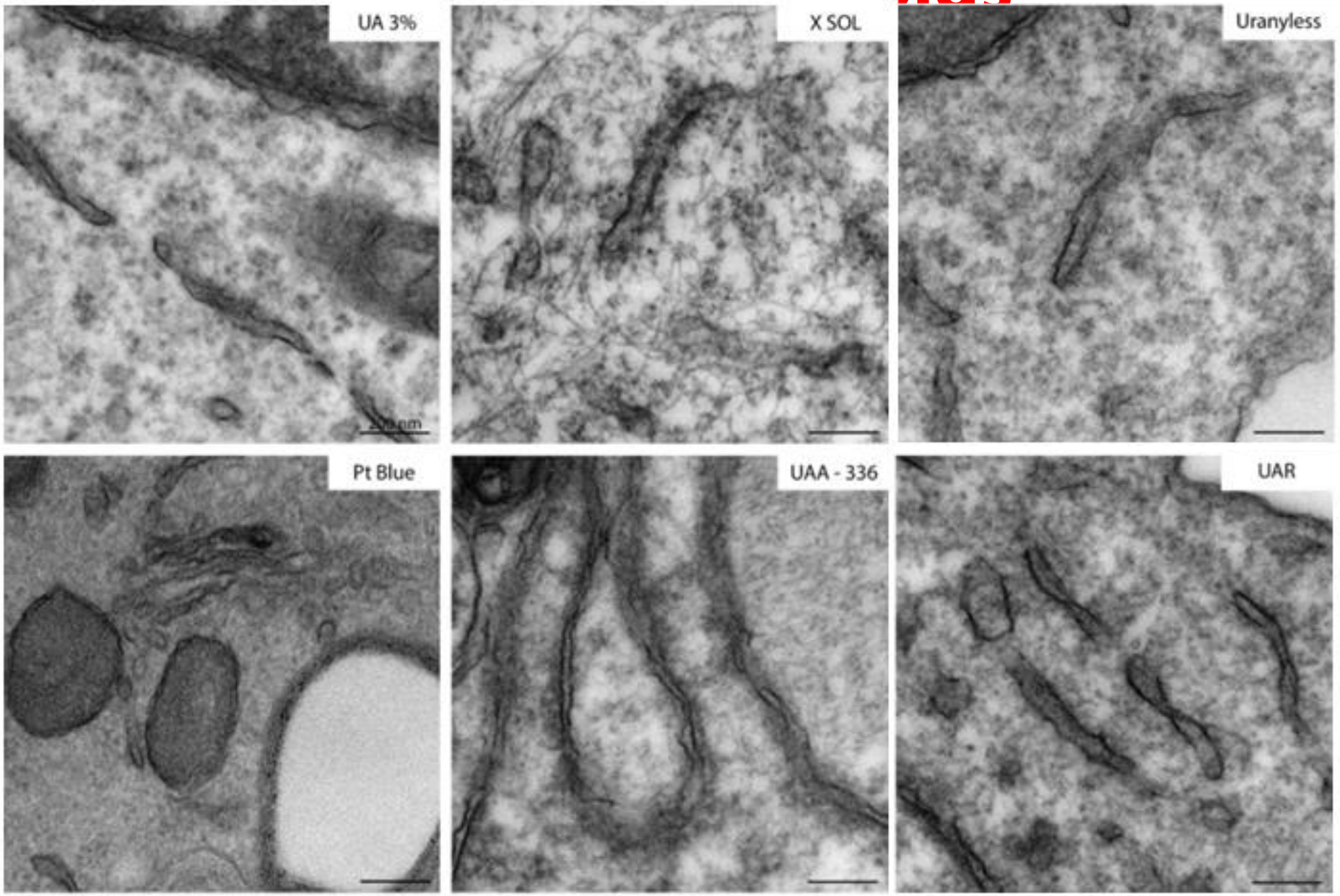

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