2nd Symposium of the Croatian Society of Clinical Embryologists and Andrology Workshop	**Workstation** **2**
Sperm morphology	

Papanicolaou staining procedure for sperm morphology

Sequentially immerse the slides in:

- 70% ethanol 10 dips
- 50% ethanol 10 dips

to rehydrate the fixed smears gradually to permit water-soluble haematoxylin staining

- Purified water 10 dips
- Haematoxylin 2 minutes *(to stain the nucleus blue)*
- Running cold tap water 2 minutes *(to remove unbound nuclear haematoxylin)*
- 0,5% HCl 3 dips *(to remove non-specifically bound dye from the cytoplasm (destaining))*
- Running cold tap water 2 minutes *(to reduce acidity and return blue colour to the nucleus)*
- Li_2CO_3 1 minute *(to neutralize the acid)*
- Running cold tap water 2 minutes
- 50% ethanol 10 dips
- 70% ethanol 10 dips
- 80% ethanol 10 dips
- 96% ethanol 10 dips

to dehydrate smears to permit ethanol-soluble Orange G/ EA-50 staining

- G-6 orange stain 3 minutes *(to stain the cytoplasm pink)*
- 96% ethanol 10 dips
- EA-50 green stain 2 minutes *(to stain the cytoplasm pink)*
- 96% ethanol 10 dips
- 96% ethanol 10 dips
- 100% ethanol 10 dips
- 100% ethanol 10 dips

*The head is stained pale blue in the acrosomal region and dark blue in the post-acrosomal region. Excess residual cytoplasm, usually located behind the head and around the midpiece, is stained pink or red.

Mounting the stained semen smears

- Immerse the slides in 100% xylene for 5 minutes
- Allow to dry in air

- Add a small amount of canada balsam (mounting medium) to the slide
- Put the slide on a hot plate for canada balsam to melt
- Place a coverslip directly on the smear
- Press gently on the top of the coverslip to help move bubbles to the edge of the slide
- Allow the mounted smear to dry

*The refractive index (RI) of mountants after drying (1.50–1.55) is similar to that of glass (1.50–1.58), and the best optical quality comes with the use of immersion oil with a similar RI (1.52).

Preparation of semen smears for computer-aided sperm morphology assessment

a) Mix the semen sample well and remove an aliquot of semen (1ml).

b) Wash to reduce background for computer-aided sperm morphology assessment:

- Dilute an aliquot of semen (1 ml) to 5 ml with normal saline (0.9 g of sodium chloride (NaCl) per 100 ml of purified water) at room temperature.
- Centrifuge at 2000 rpm for 10 minutes.
- Carefully aspirate and discard the supernatant.
- Resuspend the pellet in ~200 µl of normal saline (depending on sperm concentration and pellet quantity) by gentle pipetting.

c) Make a smear of the suspension by spreading sperm suspension on a microscope slide:

- Label the slide with identifying information using a diamond bur.
- Make a smear of the suspension by spreading 5–10 µl of sperm suspension (depending on sperm concentration) over the surface of the slide by pushing the horizontal pipette.
- Allow the slides to dry in air (10 min) and fix them in 96% ethanol (for at least 15 minutes).

d) Stain the slides using the Papanicolaou procedure.

Figure 1 Making a smear

Computer-aided sperm morphology assessment

- Add a drop of immersion oil on the stained and mounted slide of semen smear
- Examine the slide at ×1000 magnification in a program Dimensions on the Hamilton Thorne device
- Evaluate at least 100 spermatozoa

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