2nd Symposium of the Croatian Society of Clinical Embryologists and Andrology Workshop	Workstation
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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<sub>2</sub>CO<sub>3</sub> 1 minute (to neutralize the acid)
- Running cold tap water 2 minutes
- 50% ethanol 10 dips
- 70% ethanol 10 dips
- 80% ethanol 10 dips
- to dehydrate smears to permit ethanol-soluble Orange G/ EA-50 staining
- 96% ethanol 10 dips
- 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.

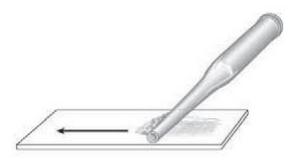


Figure 1 Making a smear

- > 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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