Methyl Methanesulfonate

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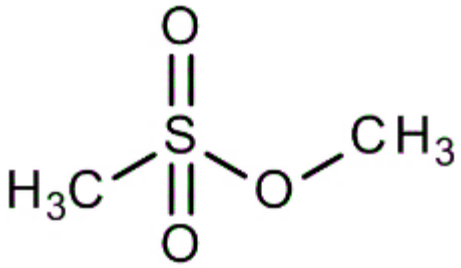
Structure and function

Methanesulfonic acid methyl ester



- **MMS is an alkylating agent used in cancer treatment**
- **Methylates DNA predominantly on N7-deoxyguanosine and N3-deoxyadenosine, and to a much lesser extent also other oxygen and nitrogen atoms in DNA bases including the phosphodiester linkage**
- **Stalls replication forks**

Structure and function

- MMS is an alkylating agent used in cancer treatment
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- Stalls replication forks

Positive control study: Design

- 5-day repeated dose toxicity study in male Wistar Crl:WI(Han) rats (age at study start: approximately 13 weeks)
- 5 animals treated orally by gavage with MMS (Aldrich): 40 mg/kg body weight in water for injection (5 mL/kg)
- recovery period of 3 weeks

Positive control study: Evaluations

The following post-mortem examinations were performed:

- **Sperm collection from vasa deferentia**
- **Sperm collection from cauda epididymides**
- **Sperm preservation in 2.5% Glutaraldehyde**
- **Preparation of stained sperm smears (Toluidin-Blue)**
- **Fixation of testes and epididymides (Davidson's fixative)**
- **Sperm Morphology Evaluation (Smears for Light Microscopy and Laser Scan Analysis)**
- **Histopathology**

Sperm evaluation

- Different methods for quantitative sperm analysis (total count of motile (live) and non-motile (dead) sperm, motility percent, motility grade profile, pH, white blood cell count, agglutination and if necessary, vitality and fructose count)
- computer-assisted sperm analysis (CASA), sperm quality analyzer (SQA), biochemical method (MTT method) etc.
- Qualitative morphological sperm analysis, either the above techniques, or simply smears but also histological evaluation of sperm by staging
- For mechanistic considerations, ultrastructural evaluation may be more useful

Laser scanning microscopy (LSM): Olympus LEXT OLS4000

- LSM was developed for material surface evaluations
- Uses focused laser for scanning an object
- Olympus 3D Laser Scanning Microscope LEXT OLS4000 with optional possibilities of Differential Interference Contrast (DIC)
- Maximal magnifications up to x17’090
- 3D images similar to SEM
- Measurements including reverse reflectance and image slope
- Abnormalities easily detectable

Normal rat sperm by LSM

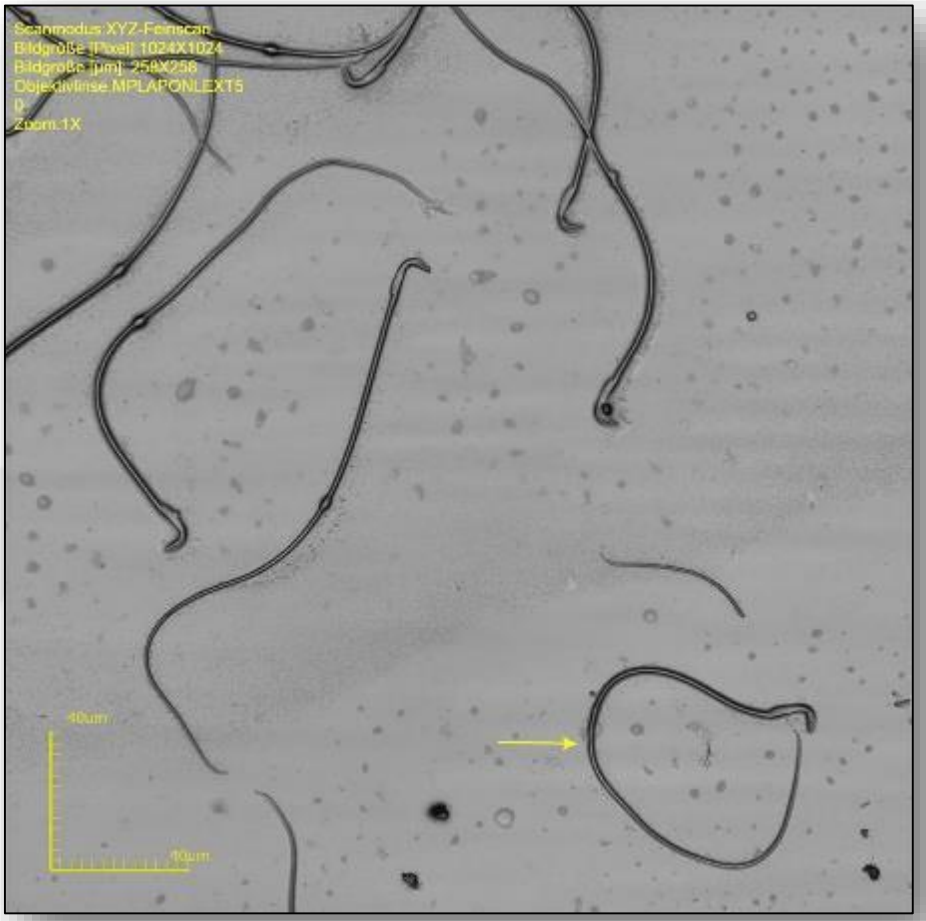
- Rodent sperm head differs (falciform type, angular) from primate sperm head (ovoid shape) and rabbit sperm (spatulate shape).
- Head divided into three different parts, anterior acrosomal segment followed by the equatorial segment and postacrosomal segment (different regions related to functional cytoskeletal changes prior to fertilization)
- Angle of approximately 90° formed by anterior acrosome
- Width at equatorial segment: 1.9 to 2.4 µm
- Lengths head: 12.2 to 16.9 µm
- Circumference:28.0 to 44.1 µm
- Head area: 24.1 to 35.0 µm2
- Tail lengths: 171.0 to 178.8 µm

Normal rat sperm

Structure	Head				Tail		Total
Param.	Length	Width	Circum-ference	Area	Length	Width	Length
Mean	15.2	2.1	38.8	28.4	175.1	1.0	190.3
SD	1.0	0.1	2.2	2.4	1.4	0.1	1.7
SD % of Mean	6.6%	5.4%	5.7%	8.3%	0.8%	6.6%	0.9%
Min	12.2	1.9	28.1	24.1	170.9	0.8	184.1
Max	16.9	2.4	44.1	35.0	178.8	1.2	193.6
Head to Tail Length Ratio (Mean)				1: 10			
Head Length %				8.0%			
Tail Length %				92.0%			

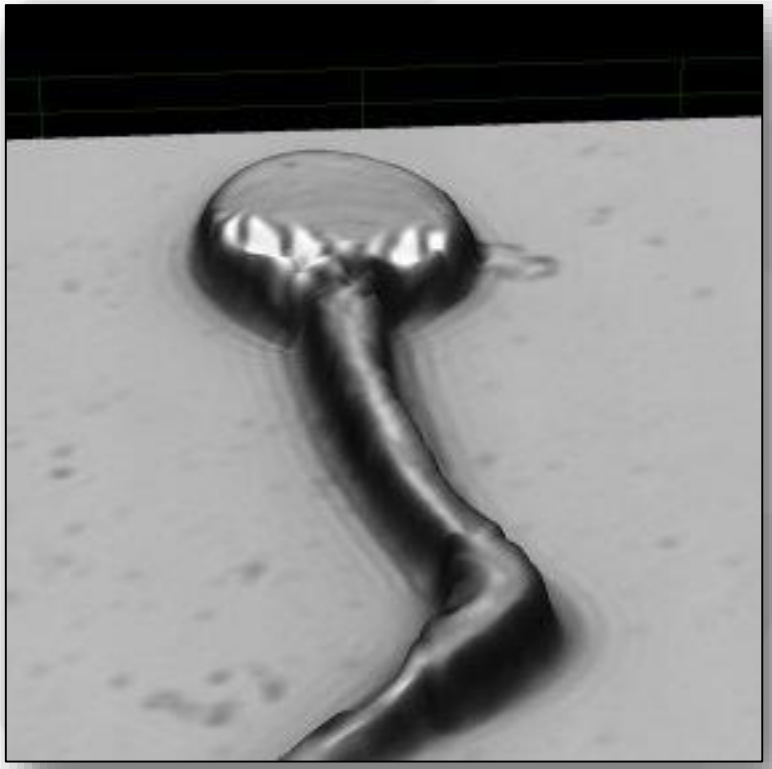
Normal rat sperm by LSM

- Almost every sperm sampled from vas deferens with cytoplasmic droplet (CD) at a mean distance of 63.4 µm distal to the head
- CD's are indicator for sperm motility and normal spermiogenesis (abnormal droplet formation, e.g. lack of droplet or ectopic droplet, indicative of defective spermiogenesis)

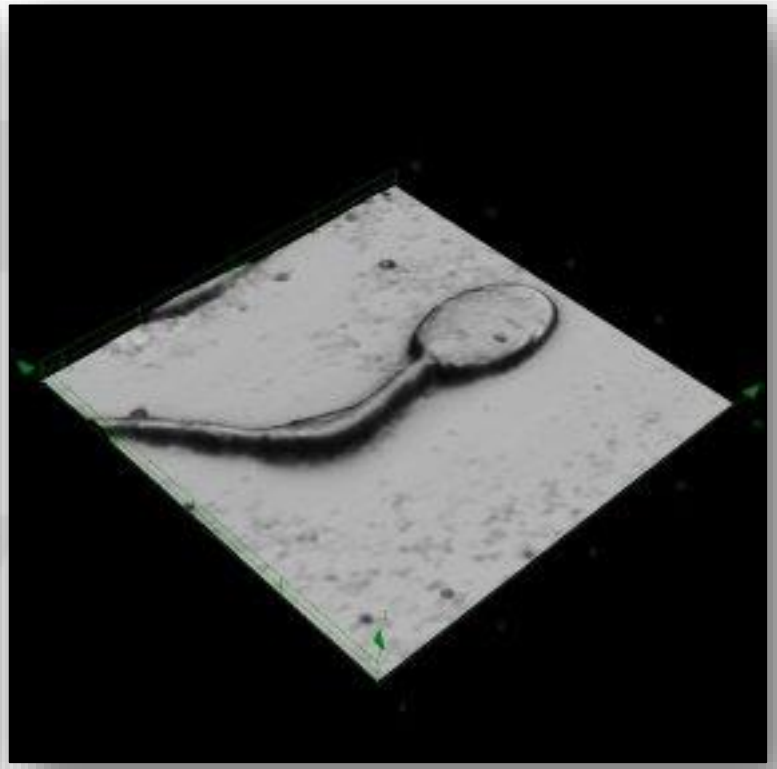


Other species

- In contrast, in primate and rabbit, >90% of sperm without CD
- If CD present, sperm is abnormal



CD in cynomolgus sperm



Cytoplasmic remnant in rabbit sperm

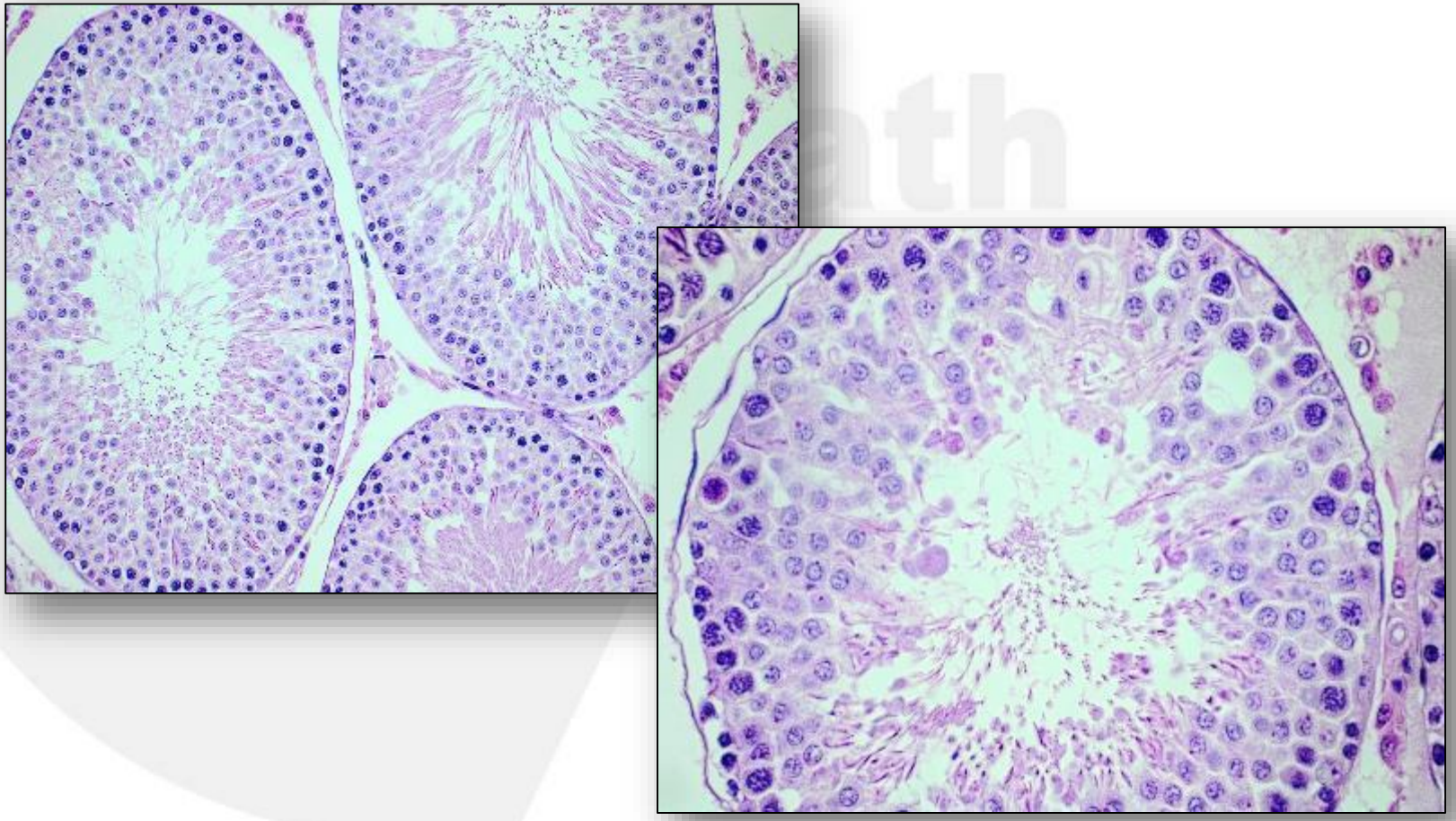
Normal rat sperm by LSM

- **Abnormalities in naïve animals do not appear often (<0.001%) include**
  missing droplet
  pyknotic sperm heads
  plasma membrane defects on tail
  curvature abnormalities
  kinks in the region of CD

- **Single heads (detached) and tails without head are common in smears and are considered to be preparation artefacts rather than abnormalities in most cases, however, with increasing abnormalities the numbers of single heads/tails increases**

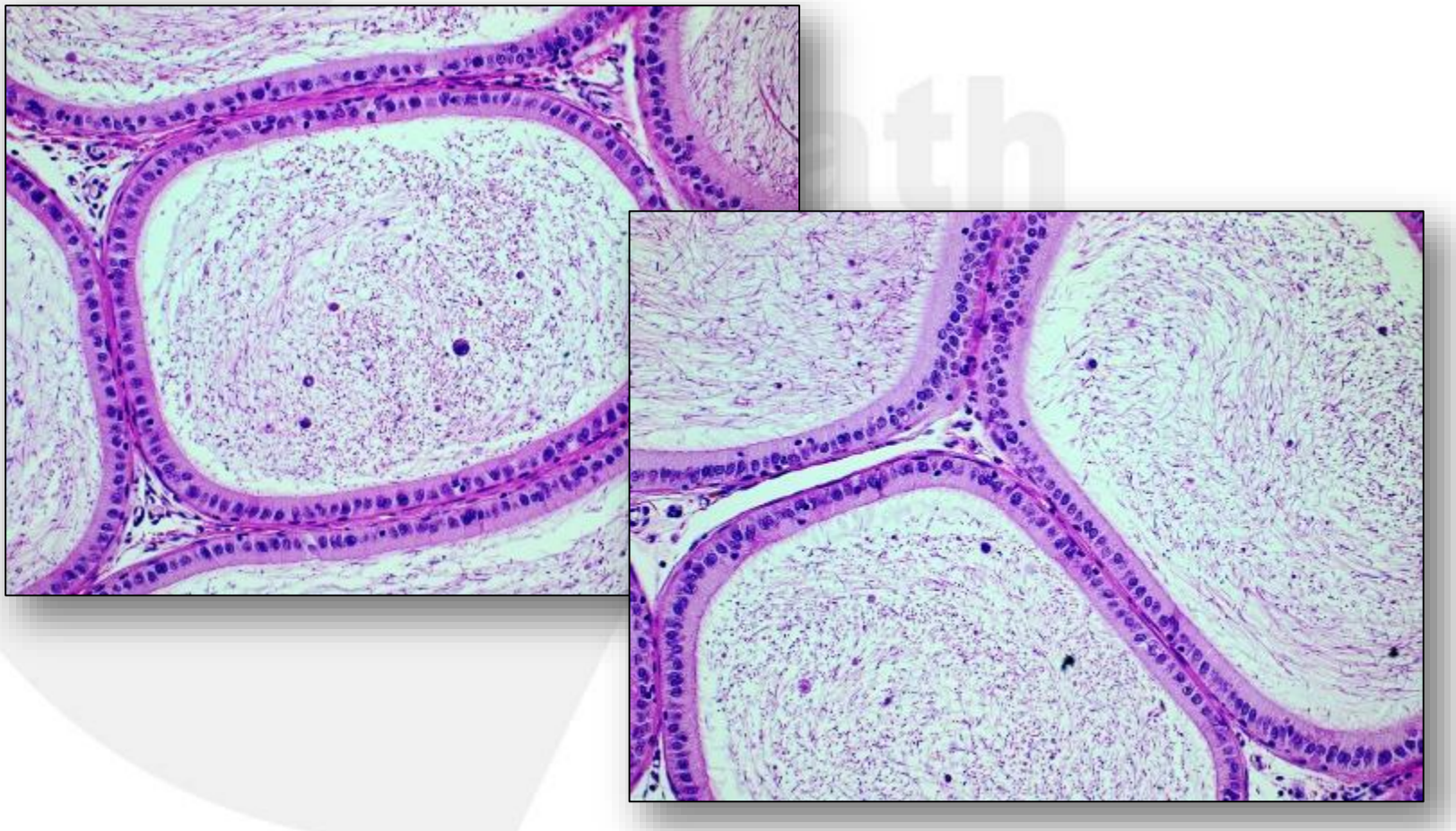
MMS-Testes: histology

- **Sperm stages in testes were complete in all animals**
- **No clear indicator for induced maturation arrest, increased resorption, necrosis or any other injury**



MMS-Epididymides: histology

- **Increased detritus and pyknotc sperms**

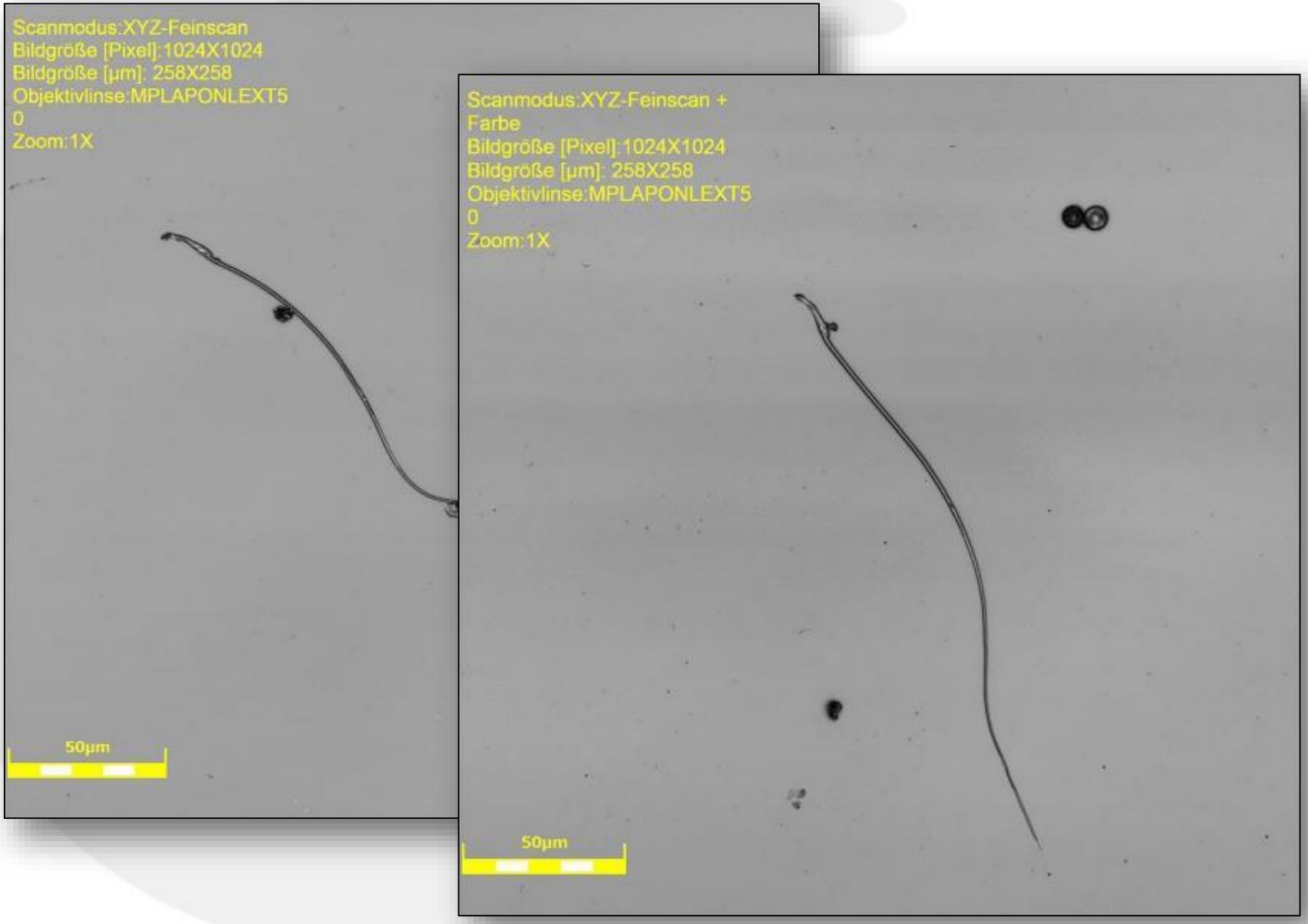


MMS-Sperm smears

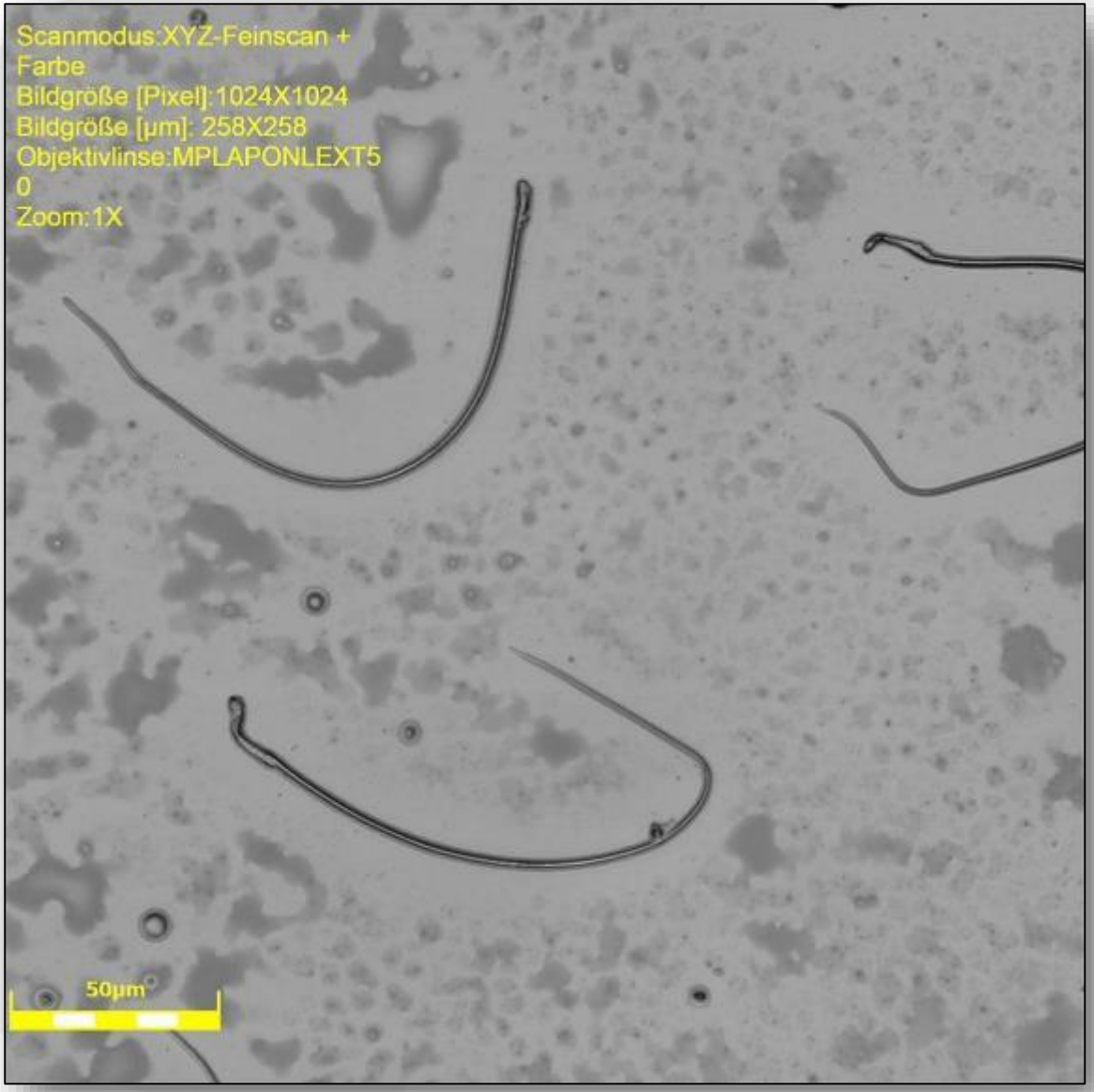
- **Vasa deferentia and cauda epididymides: abnormalities for almost all sperm cells.**

Lesion	Incidence (%) Epididymides	Incidence (%) Vas deferens	Comment
Missing Cytoplasmatic Droplet	50	82	-
Misplaced Droplet	8	3	Droplet at wrong position
Pyknosis	7	72	Pyknotic or missing heads / heads that appear to be absent under the light miscroscope
Stretched Head	40	14	Curvature Abnormality (angle approximately >115°) Many sperm cell heads stretched up to approximately 180° Extremely long and stretched heads
Total Other Abnormalities	12	17	Completely Misshapen Heads, Tail Abnormalities etc.

Curvature abnormalities: epididymides



Curvature and CD abnormalities, pyknosis : epididymides

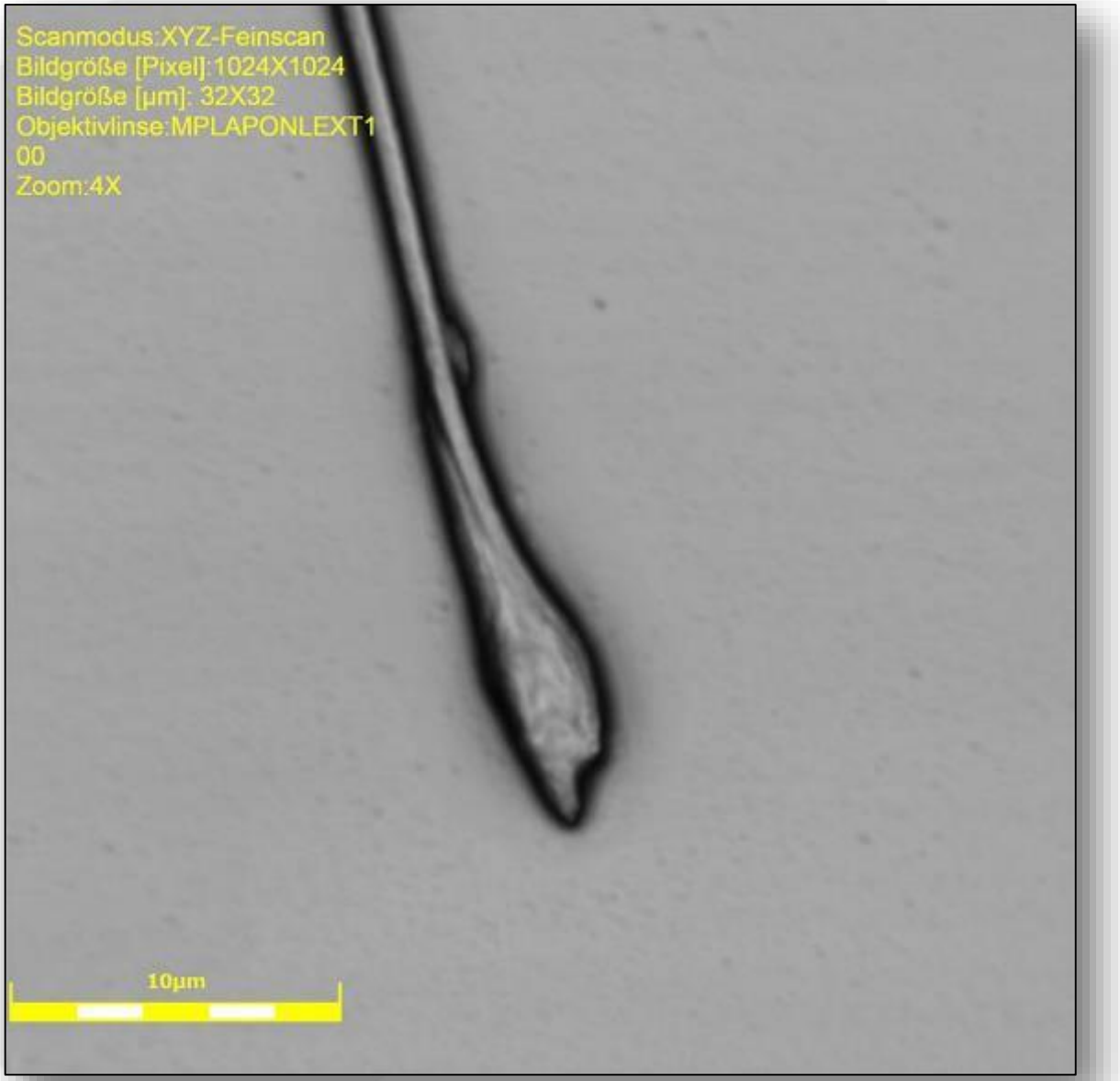


Head shape abnormalities: Vas deferens

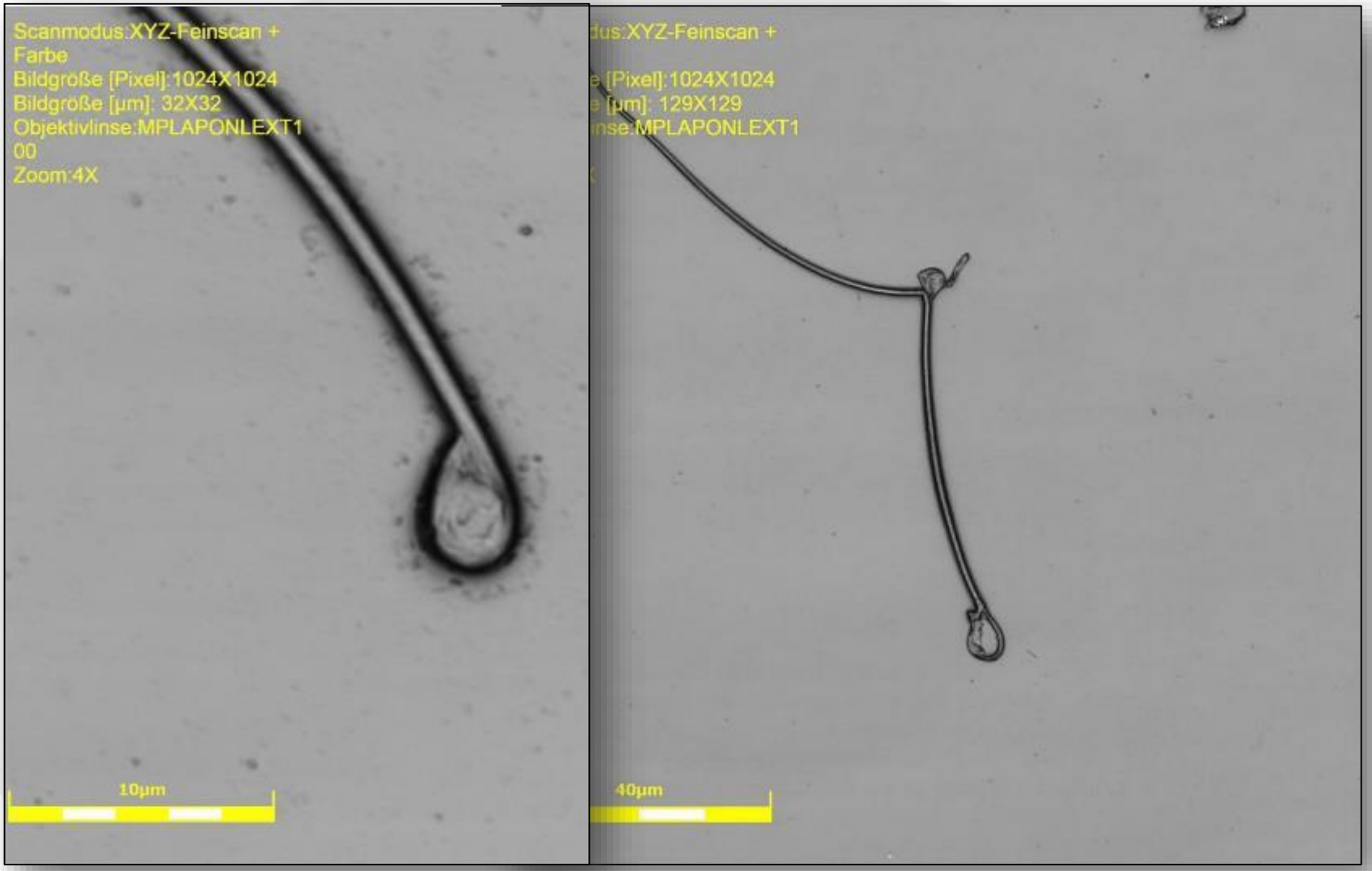
CD abnormalities: Vas deferens

Curvature abnormalities and coiled head: Vas deferens

Pyknosis: epididymides



Neck kinks and coiled heads: epididymides



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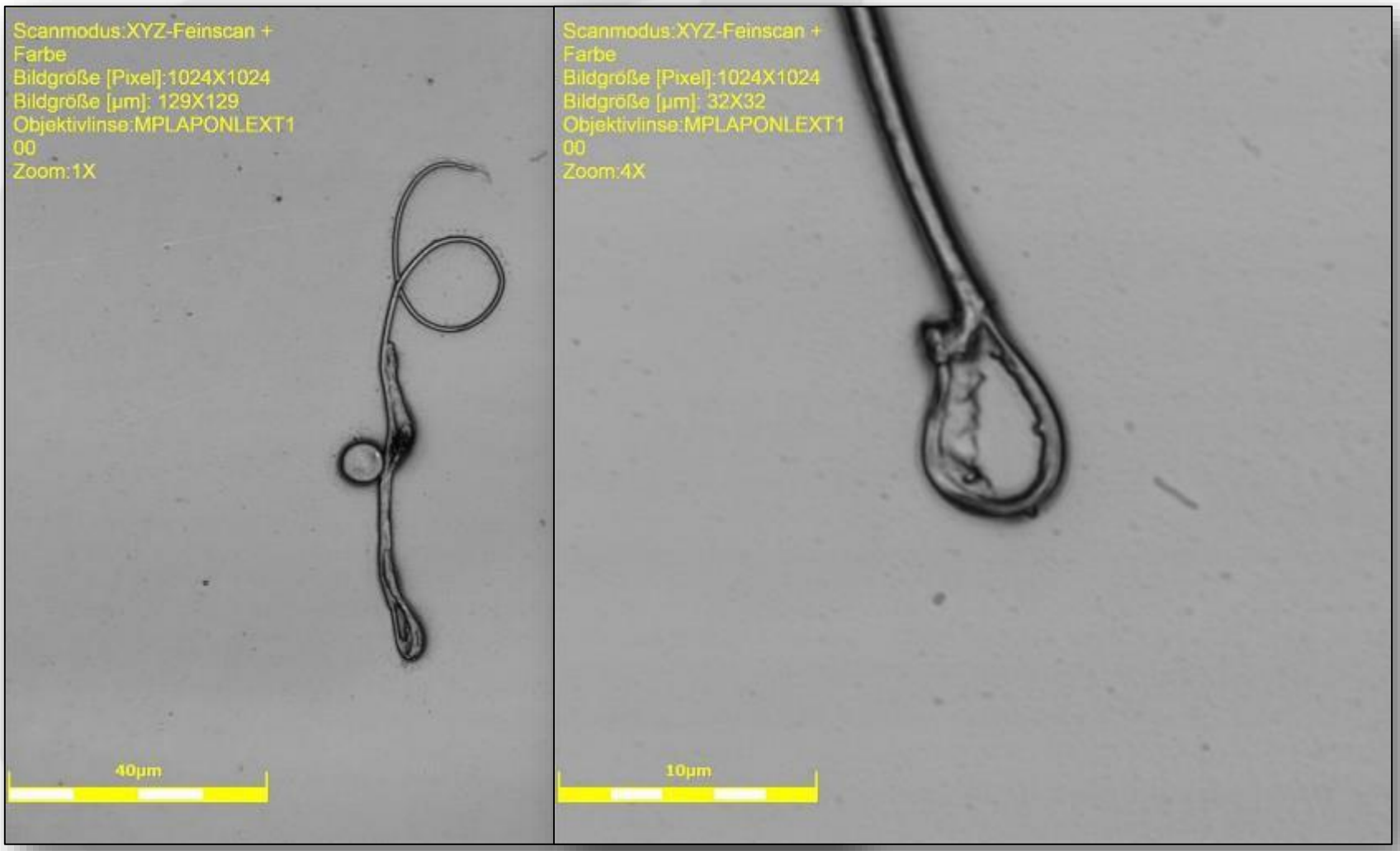
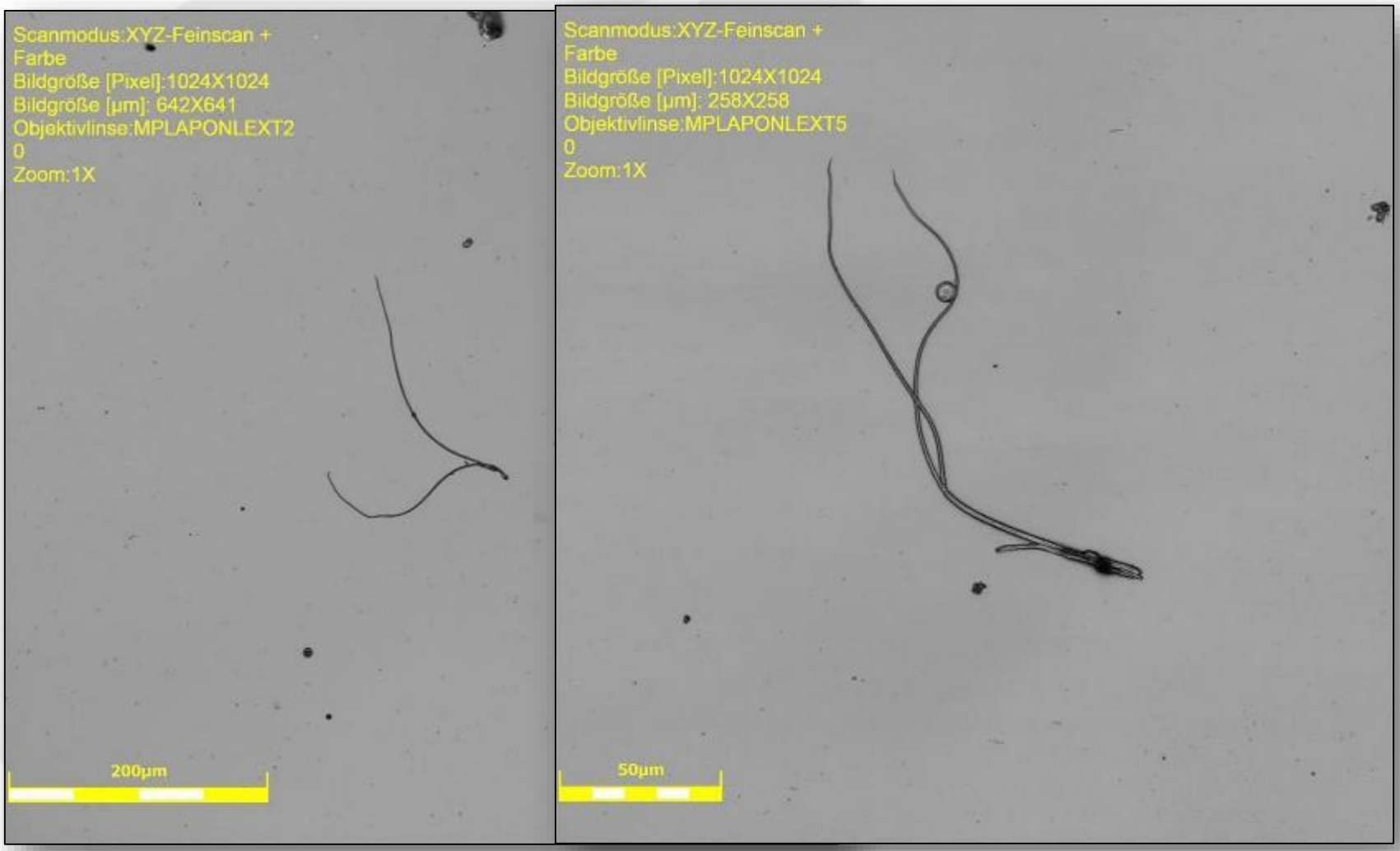
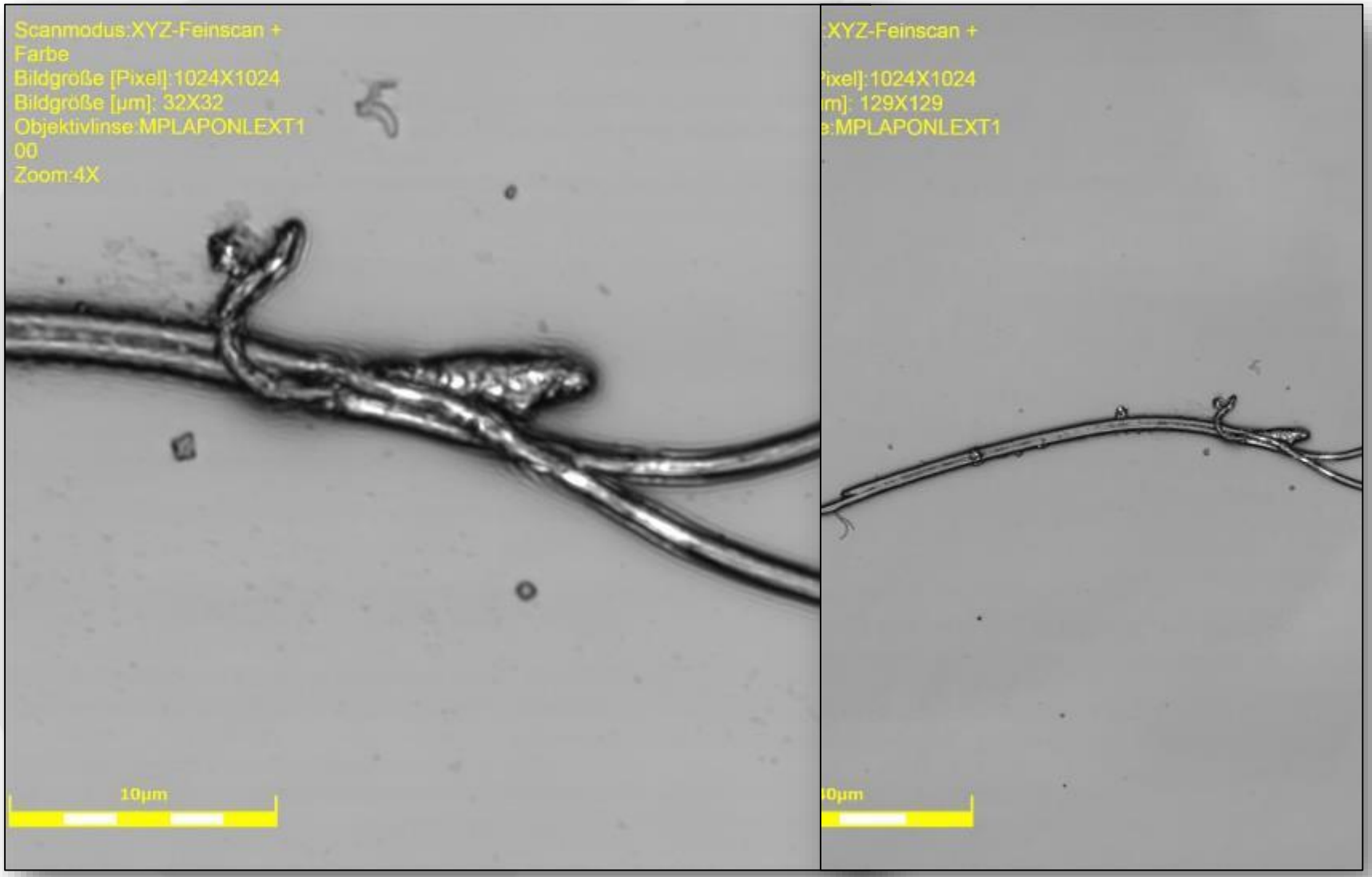


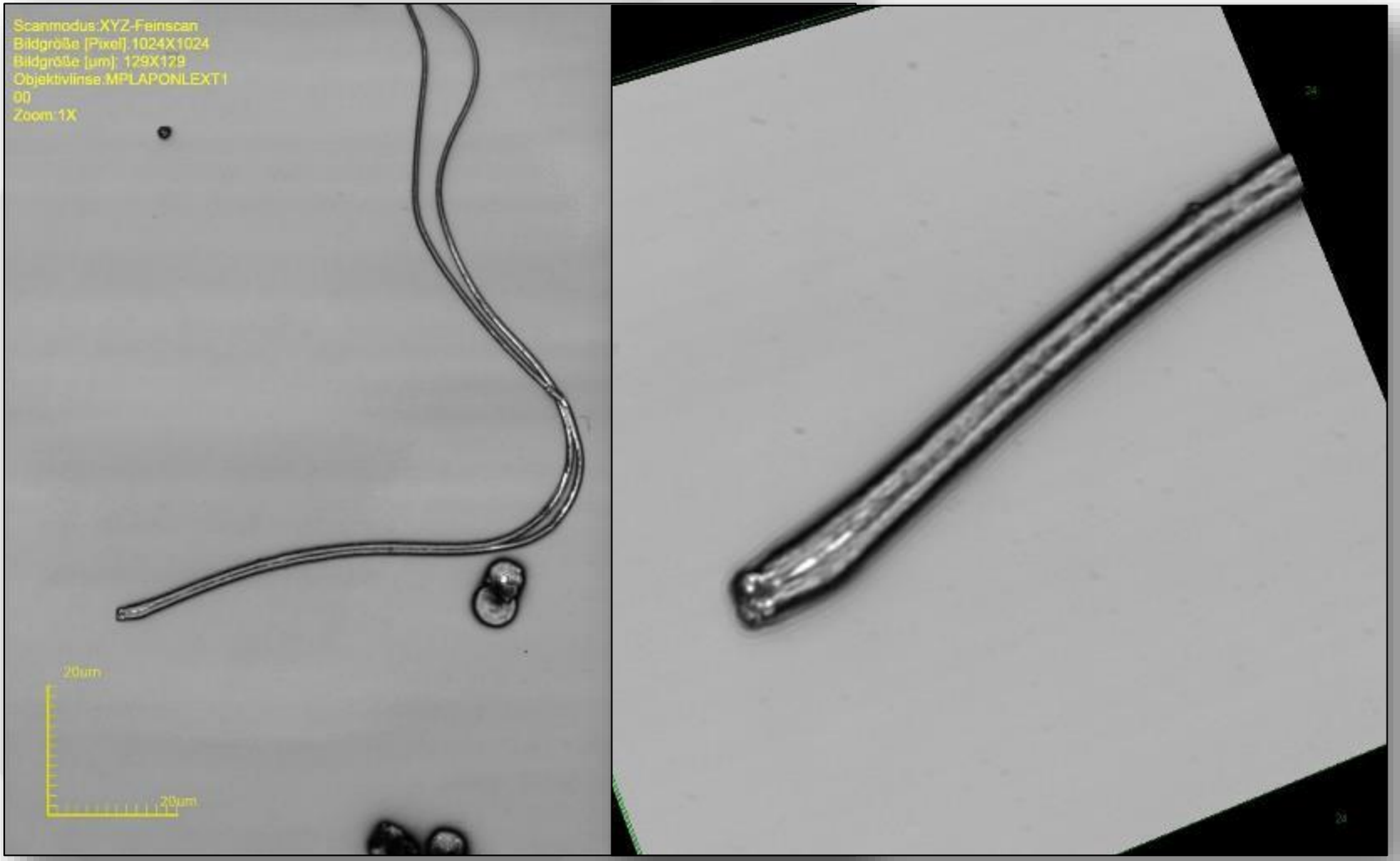
Spermatids not divided during development: epididymides



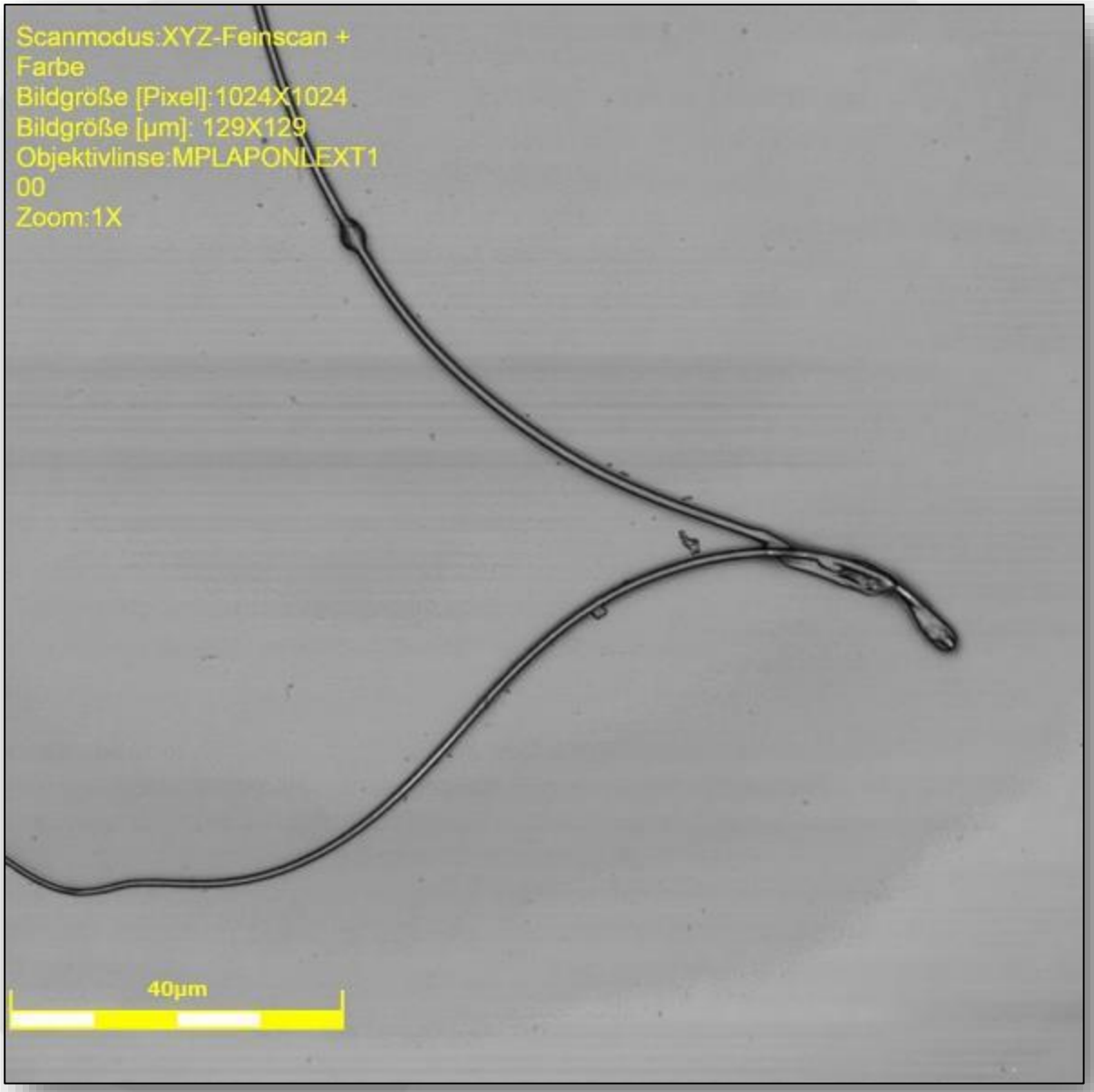
Spermatids not divided during development: Vas deferens



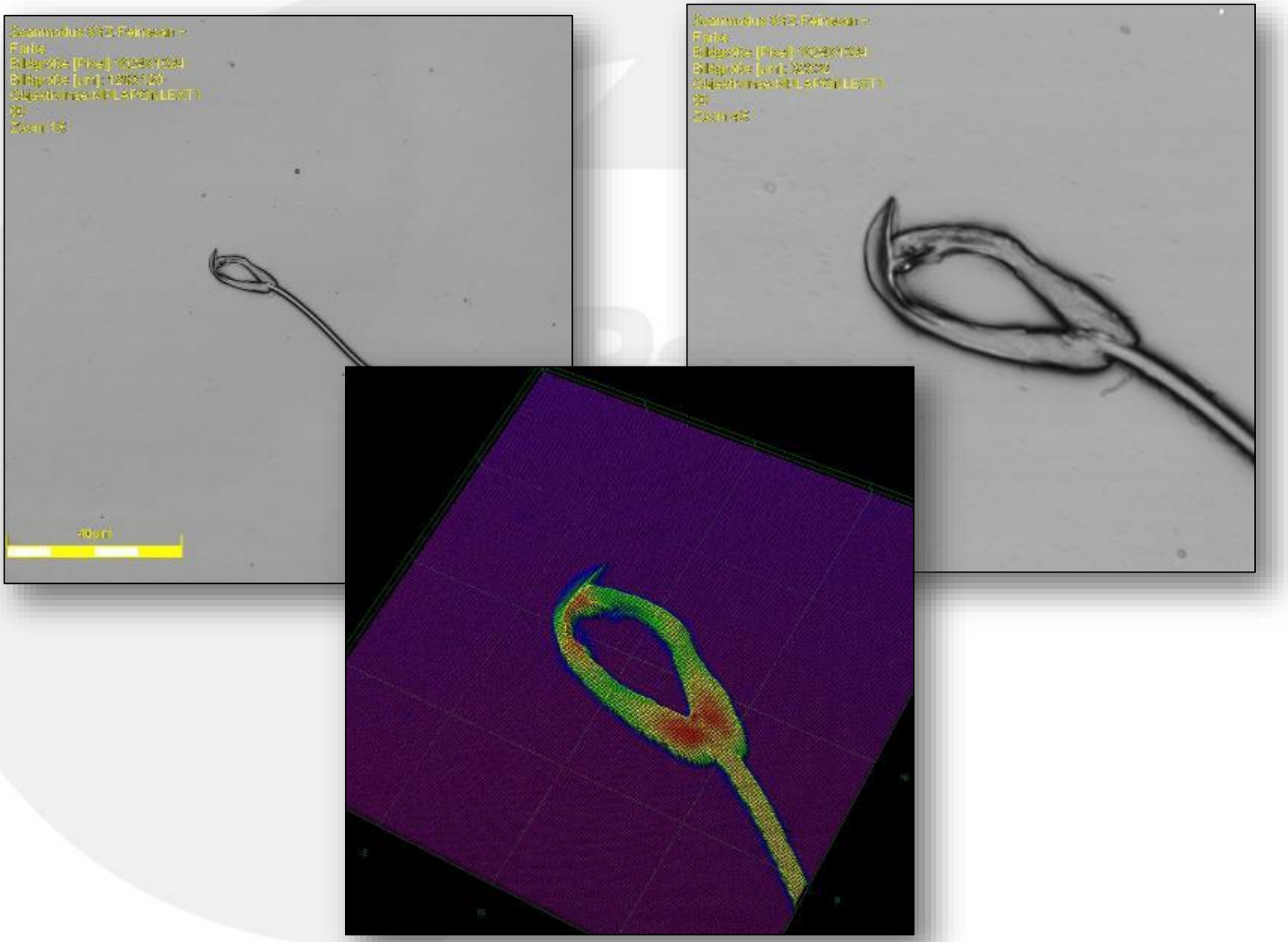
Double tailed: not separated spermatids



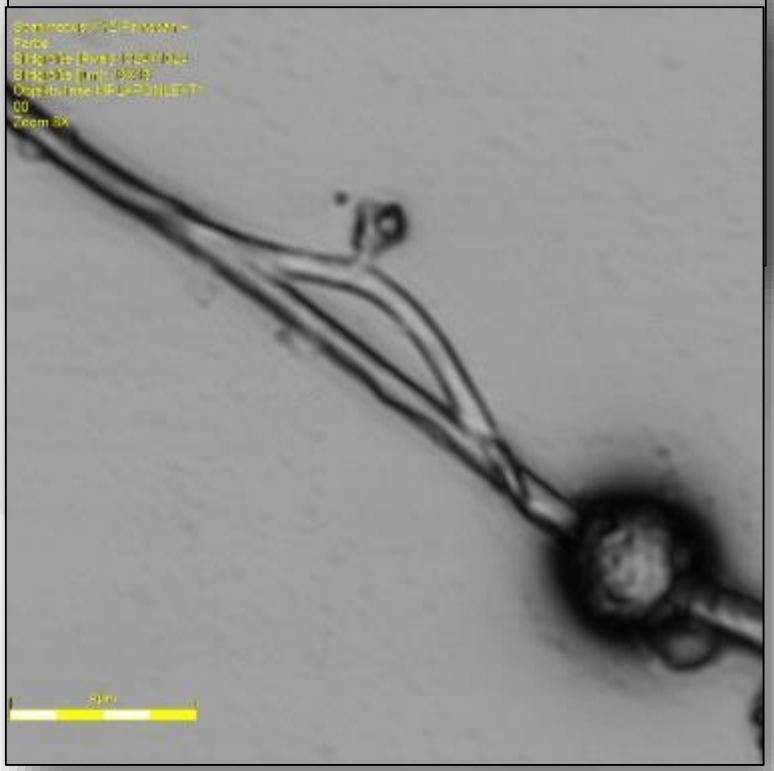
Fake double tailed sperm: epididymides

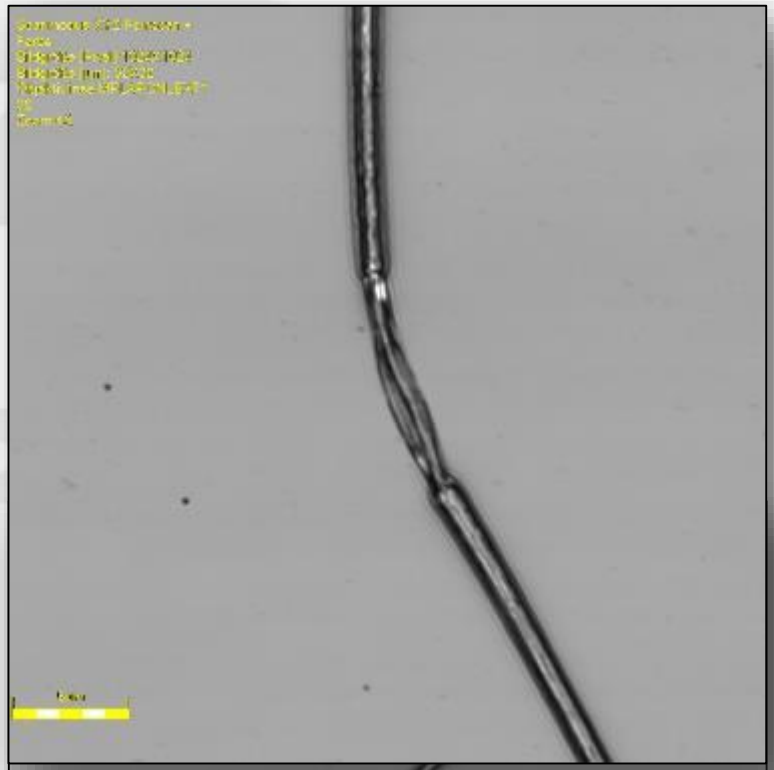


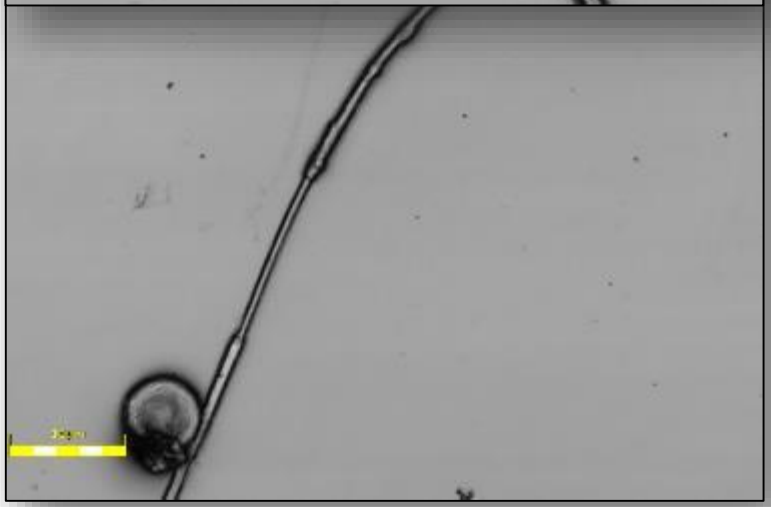
Double headed sperm: epididymides



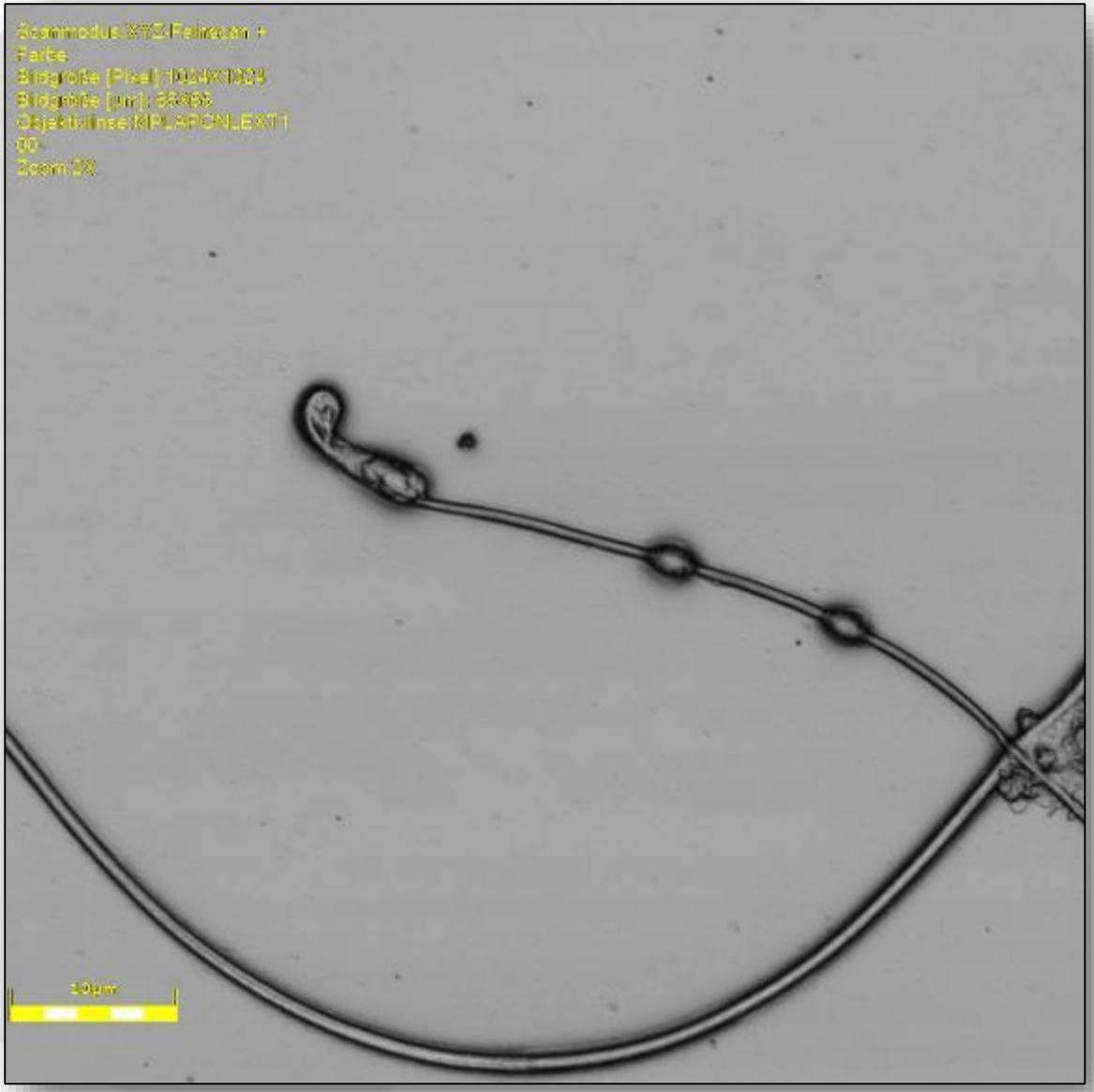
Tail abnormalities: epididymides



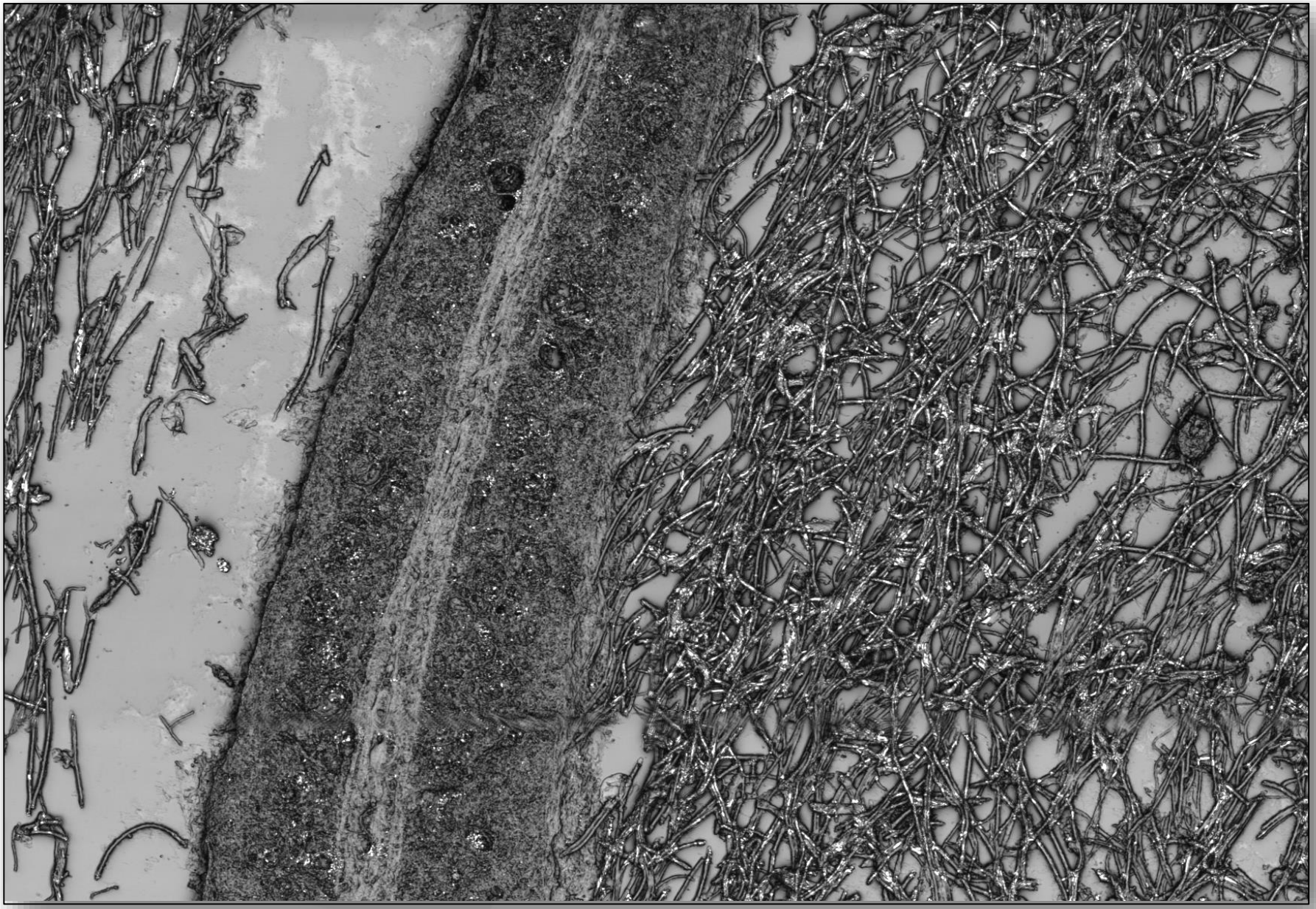




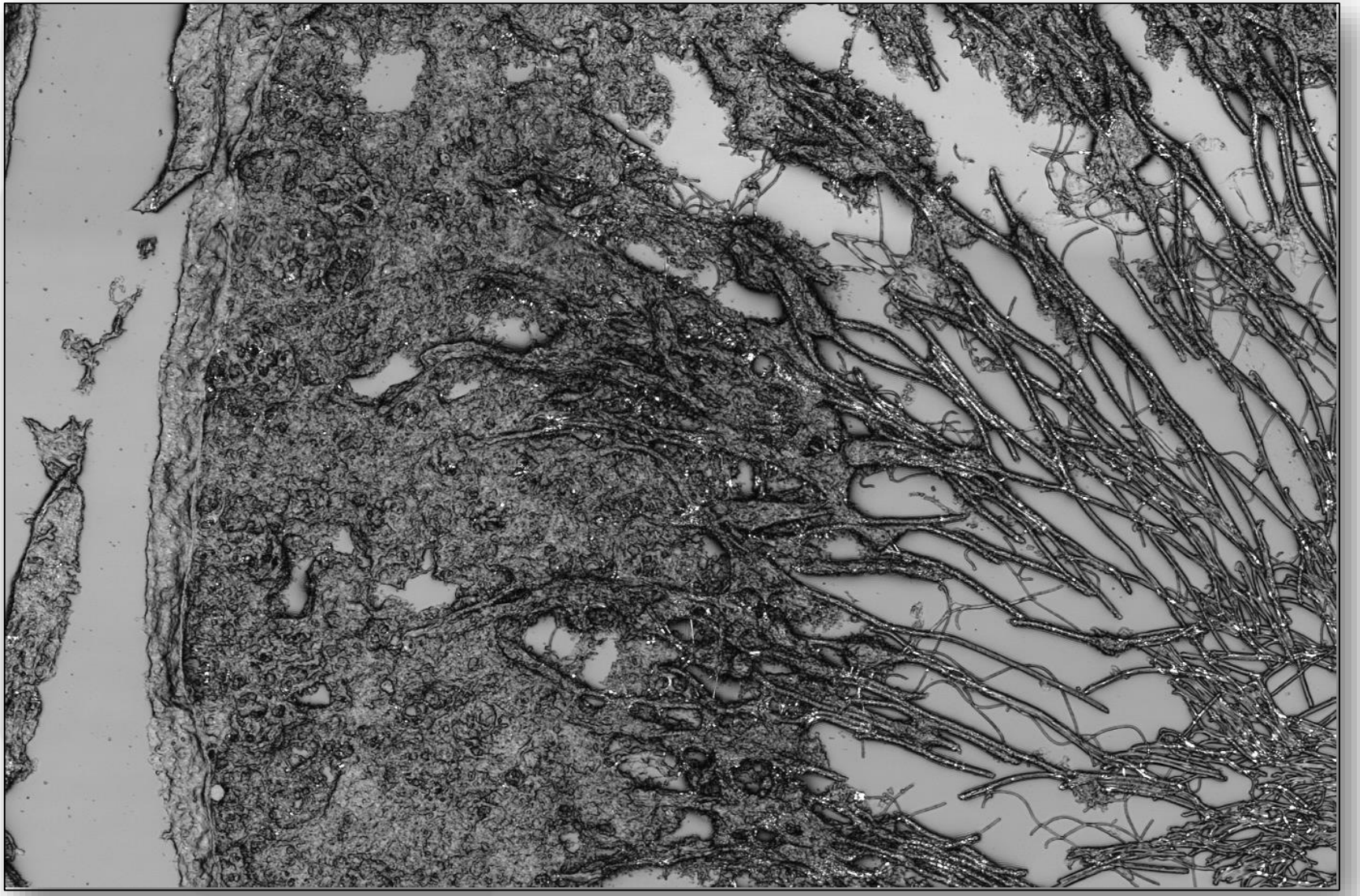
Cytoplasm membrane defect and CD‘s: epididymides



LSM: unstained section, x 2136, epididymis



LSM: unstained section, x 2136, testis



Mechanism of MMS

- MMS known for effects on rodent sperm
- Alkylation of cysteine-SH groups in sperm protamines
- Destabilization of chromatin structure causing broken chromosomes and mutations
- Curvature abnormality including abnormal stretching and thinning likely by cytoskeletal changes
- Attachments of sperms to form double-tailed/double-headed cells likely a result of tight junction damaged and incomplete separation during spermiogenesis
- Defect sperm should be partially re-absorbed by epididymal epithelium, however, this could not be demonstrated.

Summary

- **LSM revealed a wide range of abnormalities not detectable by light microscopy including head abnormalities others than curvature abnormalities consisting of:**

- **Pyknosis (round, dense, shortened head)**
- **Missing head but deposition of some material supposed to be an accumulation of cytoplasm.**
- **Head appears to be absent but is kinked**
- **Head appears to be absent but is stretched, lost its curvature and may be of a diameter similar to the tail**

Summary

- **Using LSM it turned out, that the double-tailed sperm cells that were observed by light microscopy, represented partially artifacts (by attachment of two sperm cells, whereby one sperm cell at least showed an abnormal extremely long stretched head)**
- **Another portion of these double-tailed sperms appeared to be a cytoplasmic membrane fusion of two heads or two tails.**

Use of LSM in sperm analysis

- Adequate fixation guarantizes qualitative high resolution images
- Quick interpretation of lesions at high accuracy
- 3D imaging and measurements
- High troughput compared to SEM
- High number of sample evaluation possible with reliable statistics
- Low costs compared to SEM
- Mechanistical investigations possible