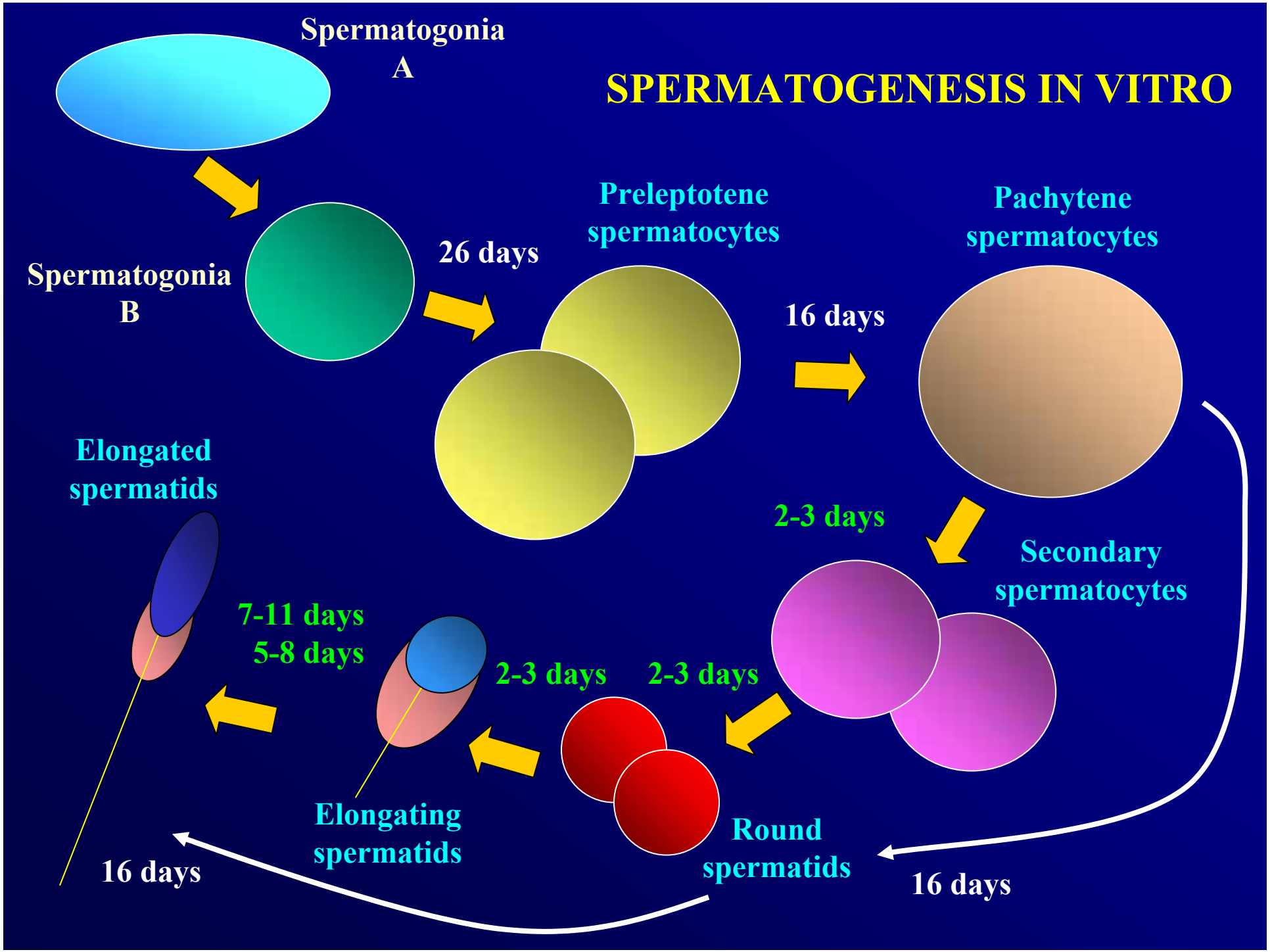
SPERMATOGENESIS IN VITRO

INDUCTION OF PROLIFERATION, MEIOSIS AND DIFFERENTIATION

Mário Sousa

Lab Cell Biology
Institute of Biomedical Sciences (ICBAS)
University of Porto
msousa@icbas.up.pt


SPERMATOGENESIS IN VITRO
Spermatogonia A
Spermatogonia B
26 days
Preleptotene spermatocytes
16 days
Pachytene spermatocytes
2-3 days
Secondary spermatocytes
2-3 days
2-3 days
Round spermatids
16 days
Elongating spermatids
7-11 days
5-8 days
Elongated spermatids
16 days

OBJECTIVES

culture medium for long term cultures and cell differentiation

cell and molecular processes at each germ cell stage

germ cell lines

homologous transplantation

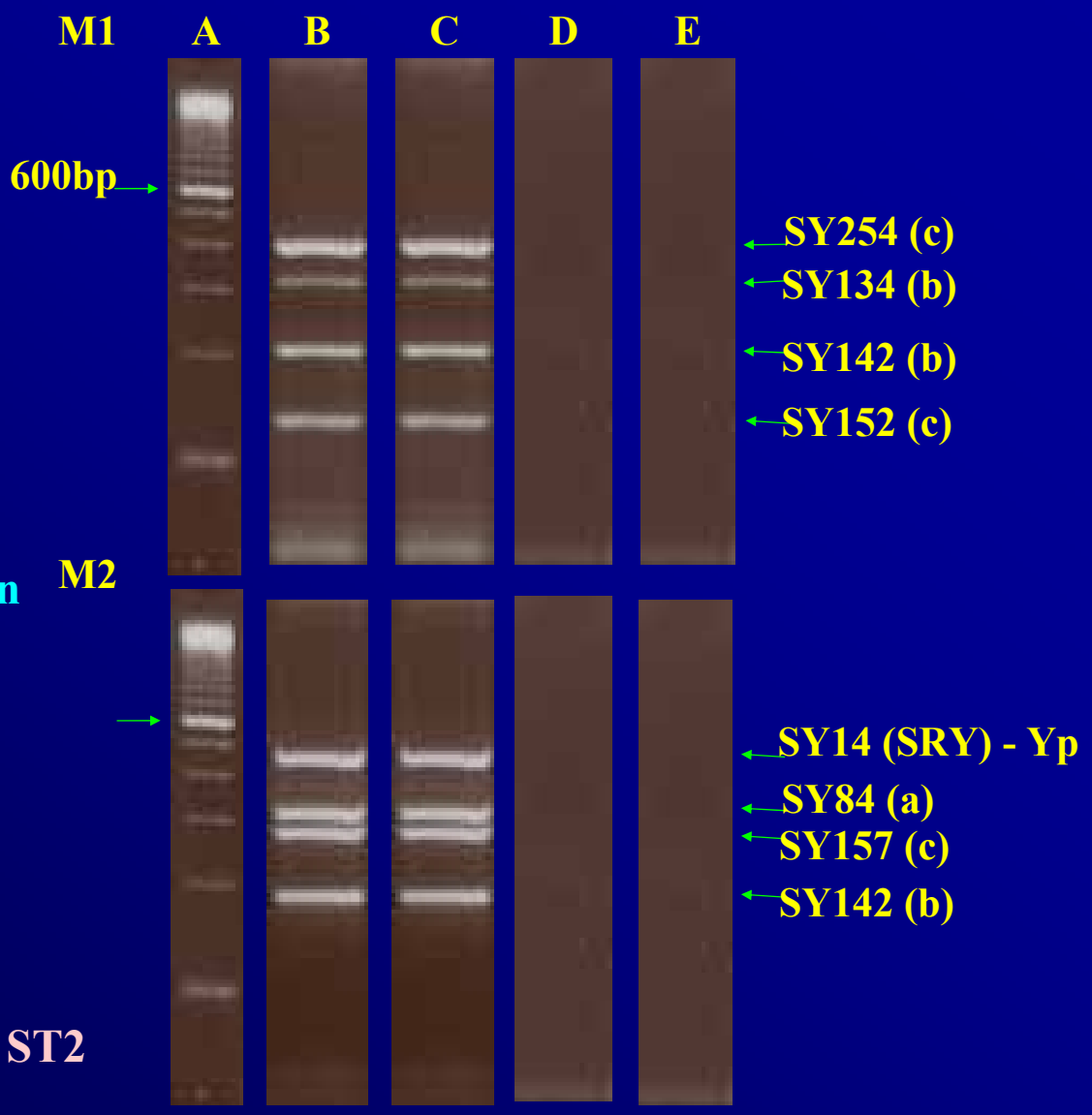
in vitro gene therapy

15 anejaculation cases
Normal karyotypes
Absence of Y microdeletions
Conserved spermatogenesis

Mechanical dissociation
Erythrocyte lysis
Enzymatic digestion
Cell isolation by micromanipulation

Cell culture:
5 CM
5 CM + rFSH (25 U/L)
5 rFSH + T (2 µmol/L)

Plated cells:
250 S + 100 SGA + 1000 ST1 + 100 ST2



Multiplex-PCR
AZF a,b,c
Yq11.2

Each testicle biopsy was collected in sperm preparation medium (SPM; Medicult, Copenhagen, Denmark) and squeezed with surgical blades.

The resultant fluid was diluted with SPM and washed by centrifuging at 1,000 rpm (500-600 g), 2 times 5 minutes.

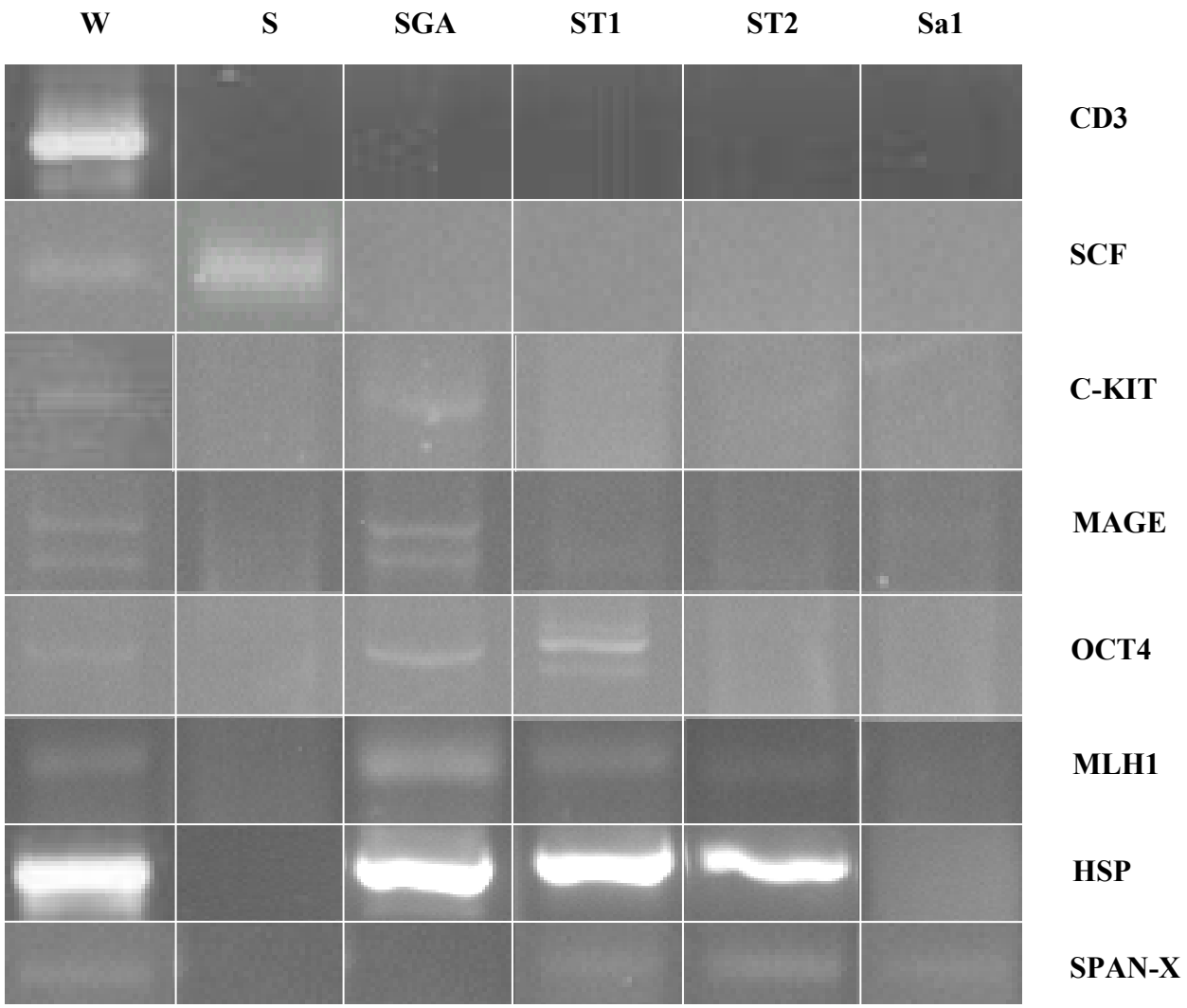
The pellet was resuspended for 5 min in 2 ml of erythrocyte-lysing buffer (Verheyen et al., 1995), prepared with 155 mM NH_4Cl, 10 mM $KHCO_3$, and 2 mM EDTA in water, pH 7.2 with KOH (all from Sigma, Barcelone, Spain, cell culture tested), and filtered by 0.2 µm.

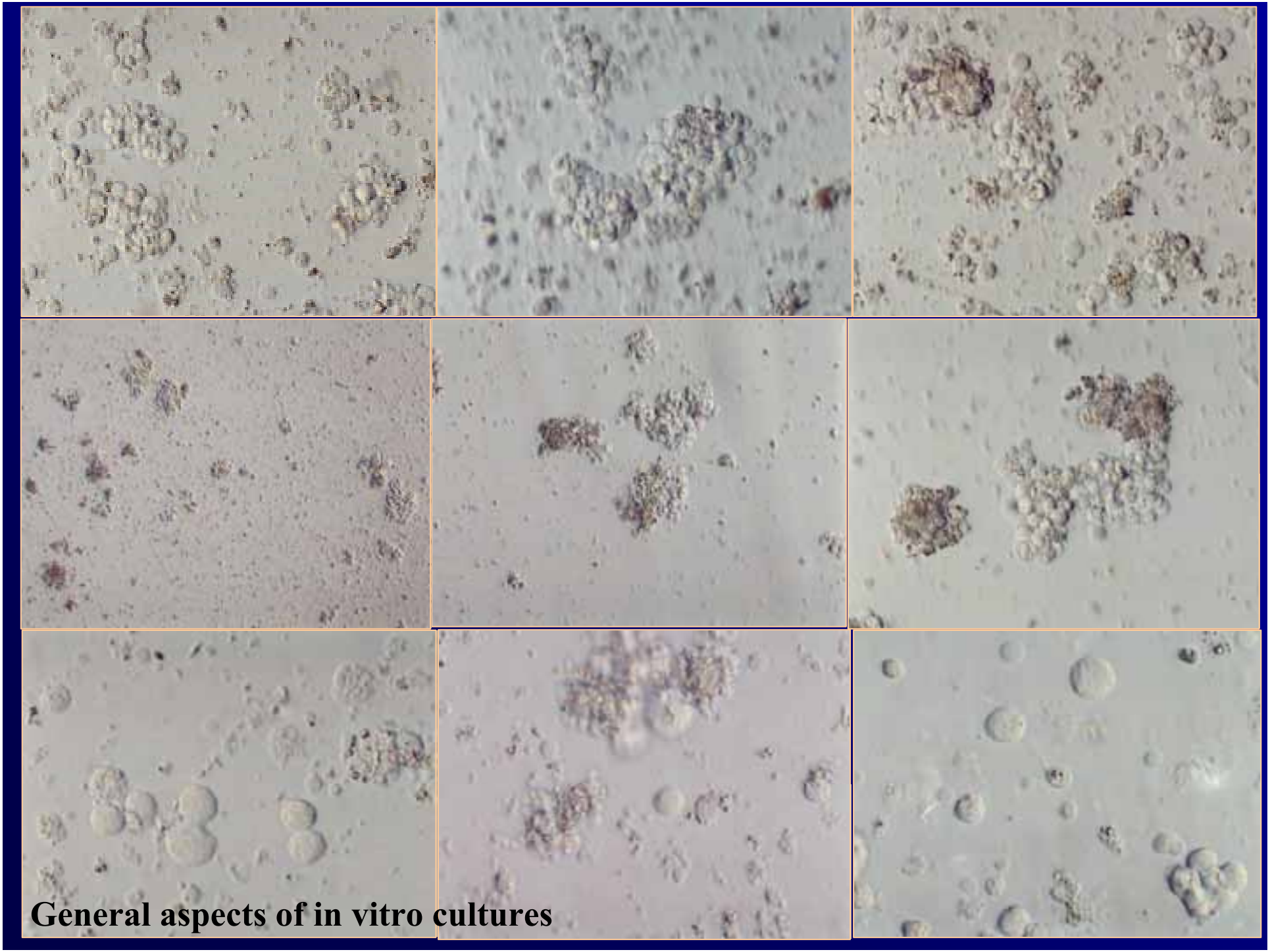
After washing, samples were digested (Crabbé et al., 1997) for 1h at 37ºC, in a solution of SPM containing 25 µg/ml of crude DNase and 1000 U/ml of collagenase-IV (Sigma).

After washing, the pellet was resuspended in IVF medium (Medicult) and incubated at 30-32ºC, 5% CO_2 in air until use.

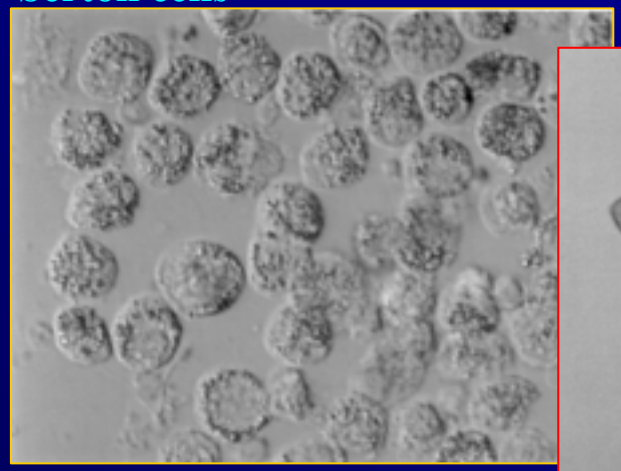
A sample was then diluted in SPM, spread on a tissue culture plate and covered with light mineral oil (Medicult).

RT-PCR: cell stages




General aspects of in vitro cultures

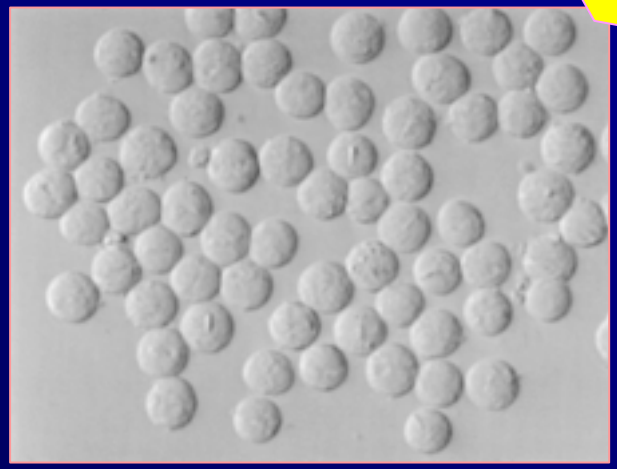
Sertoli cells



Spermatogonia A

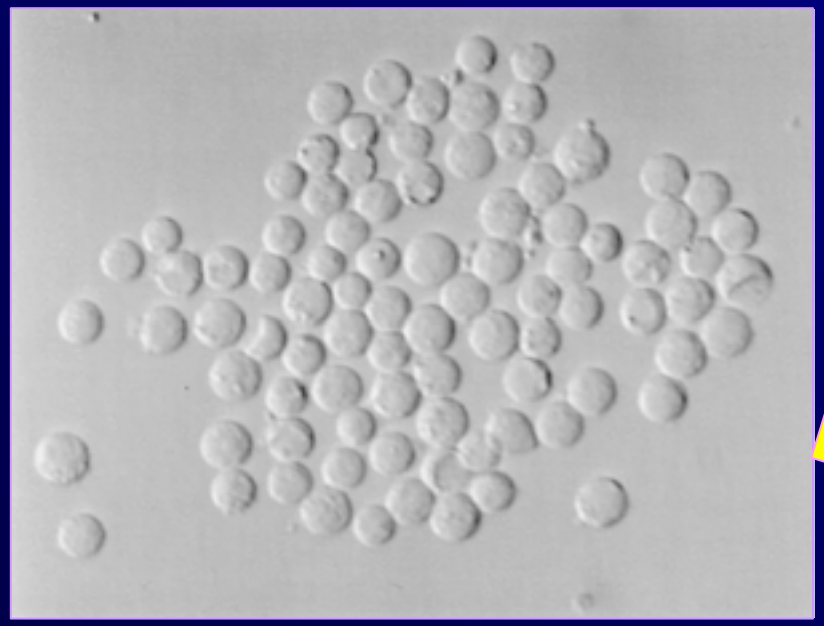
Primary spermatocytes

Secondary spermatocytes



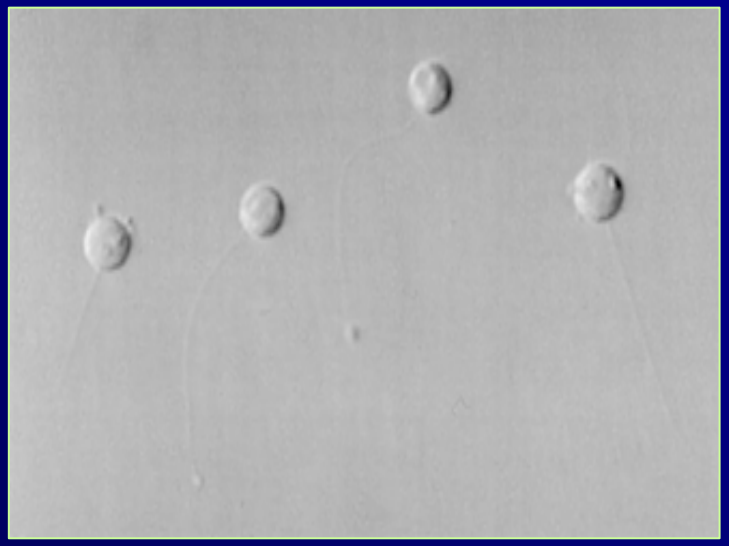
2-4 days

1-2 days

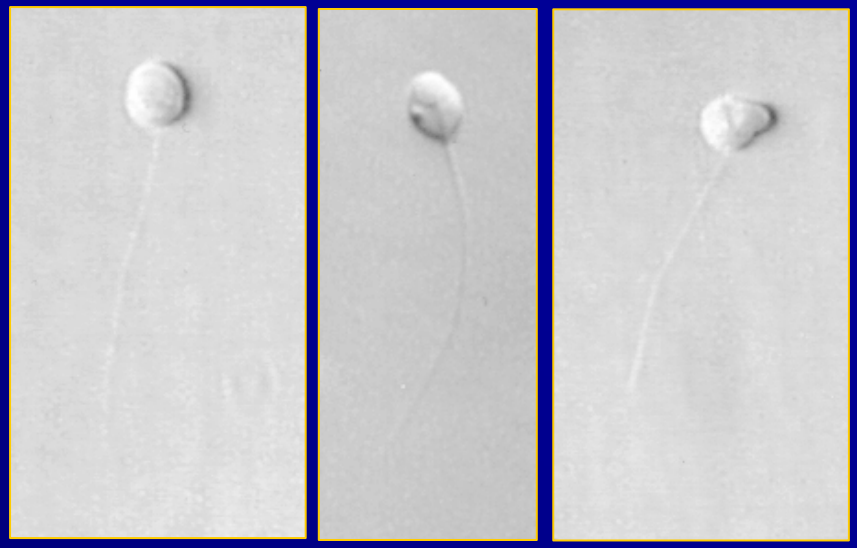


Round spermatids without tails

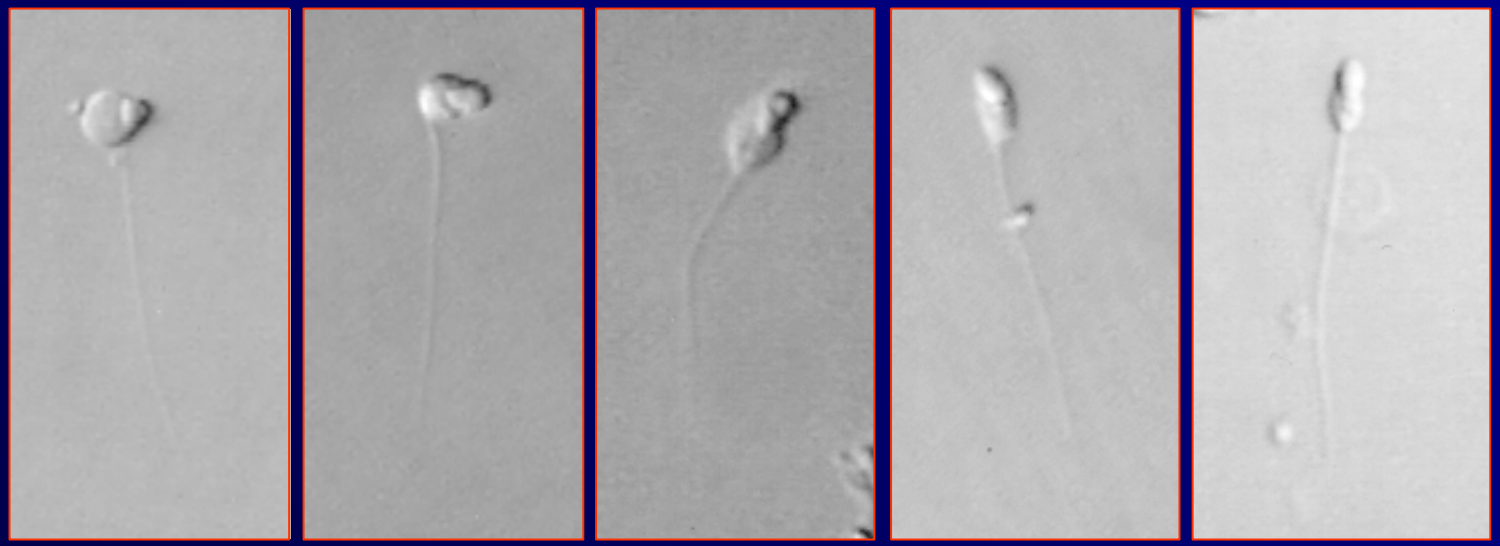
Round spermatids with tail: 4 days

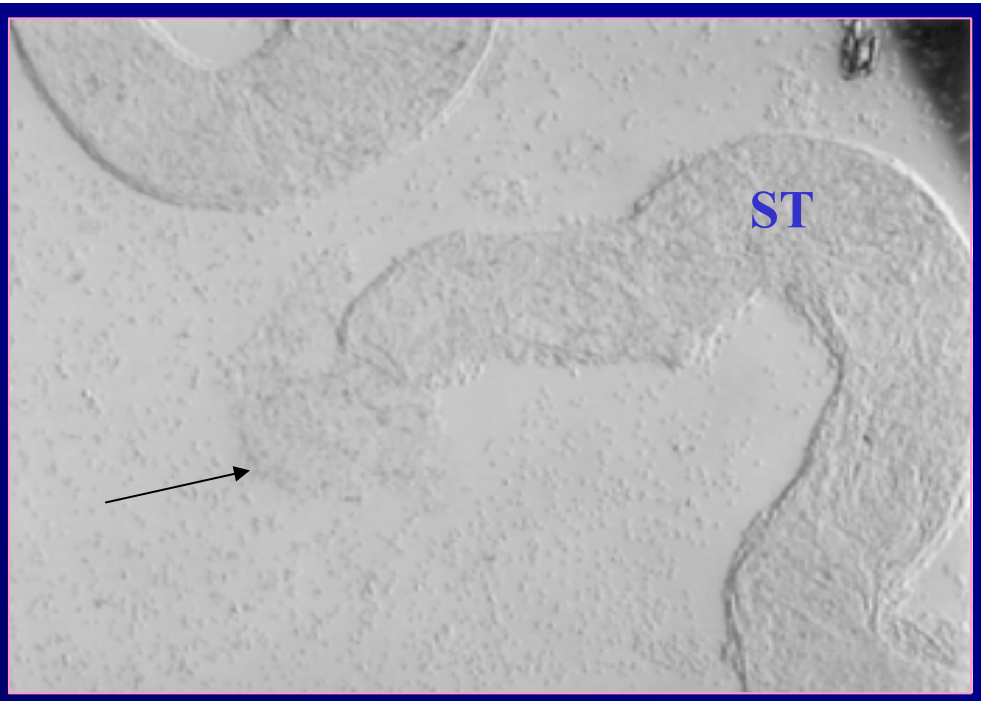


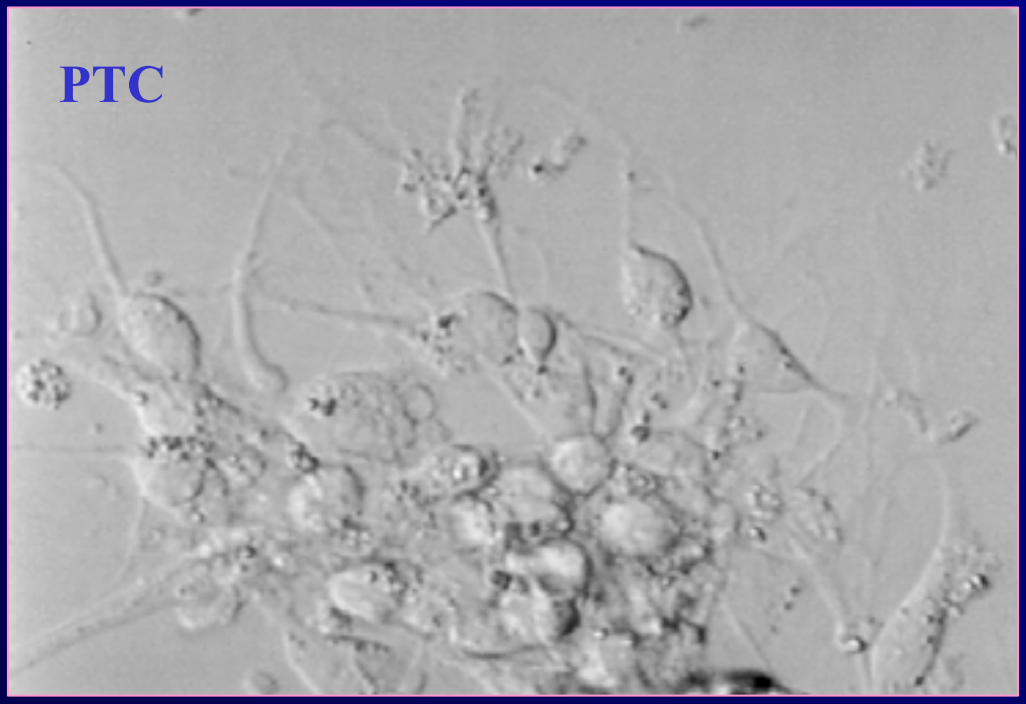
Elongating spermatids: 7 days

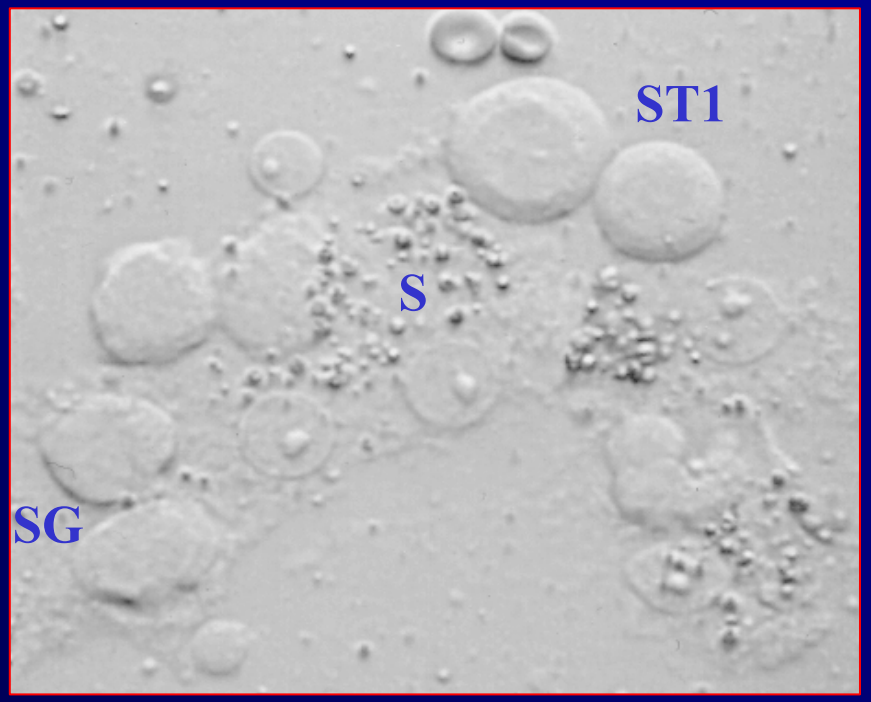


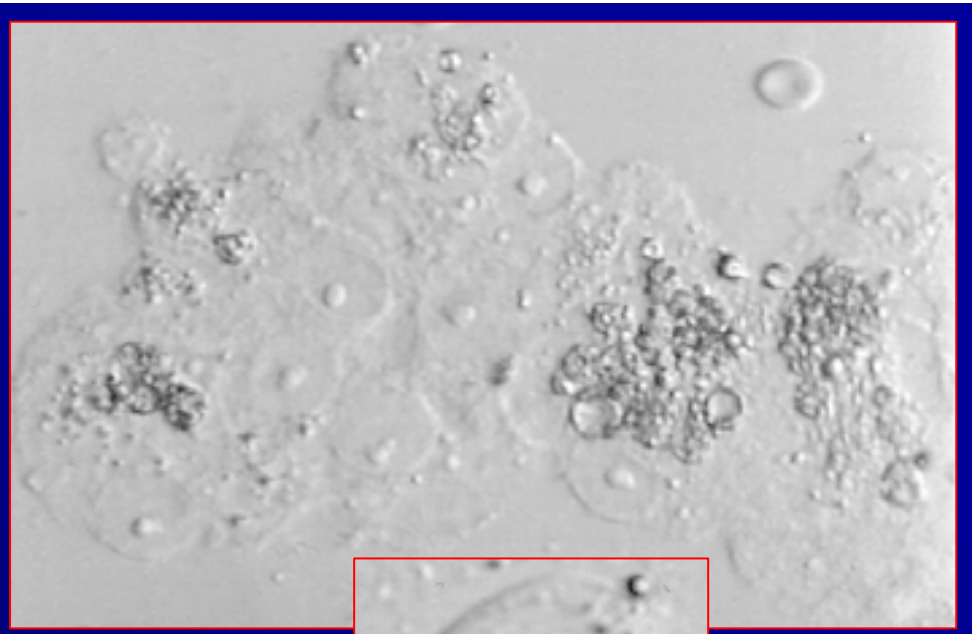
Elongated spermatids: 14 days

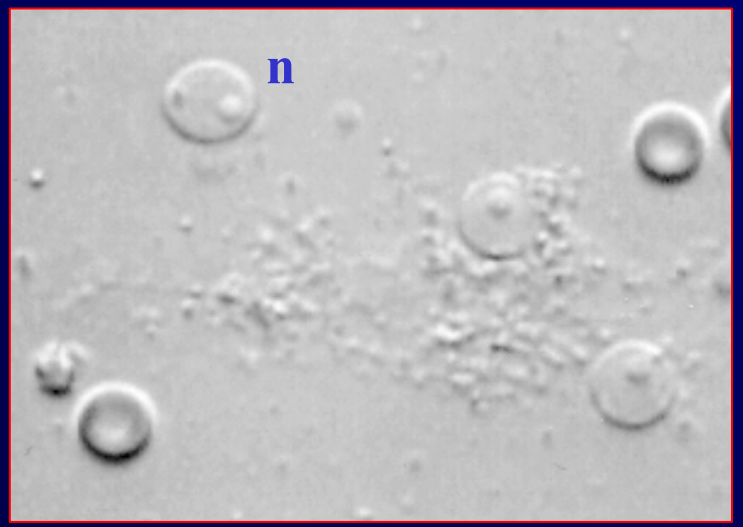


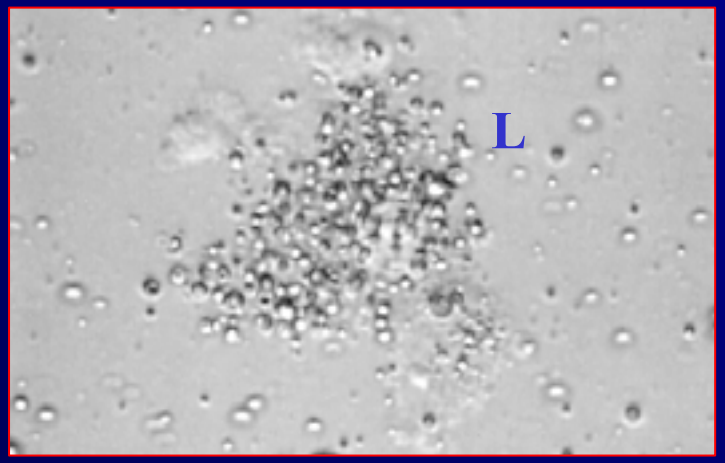

ST

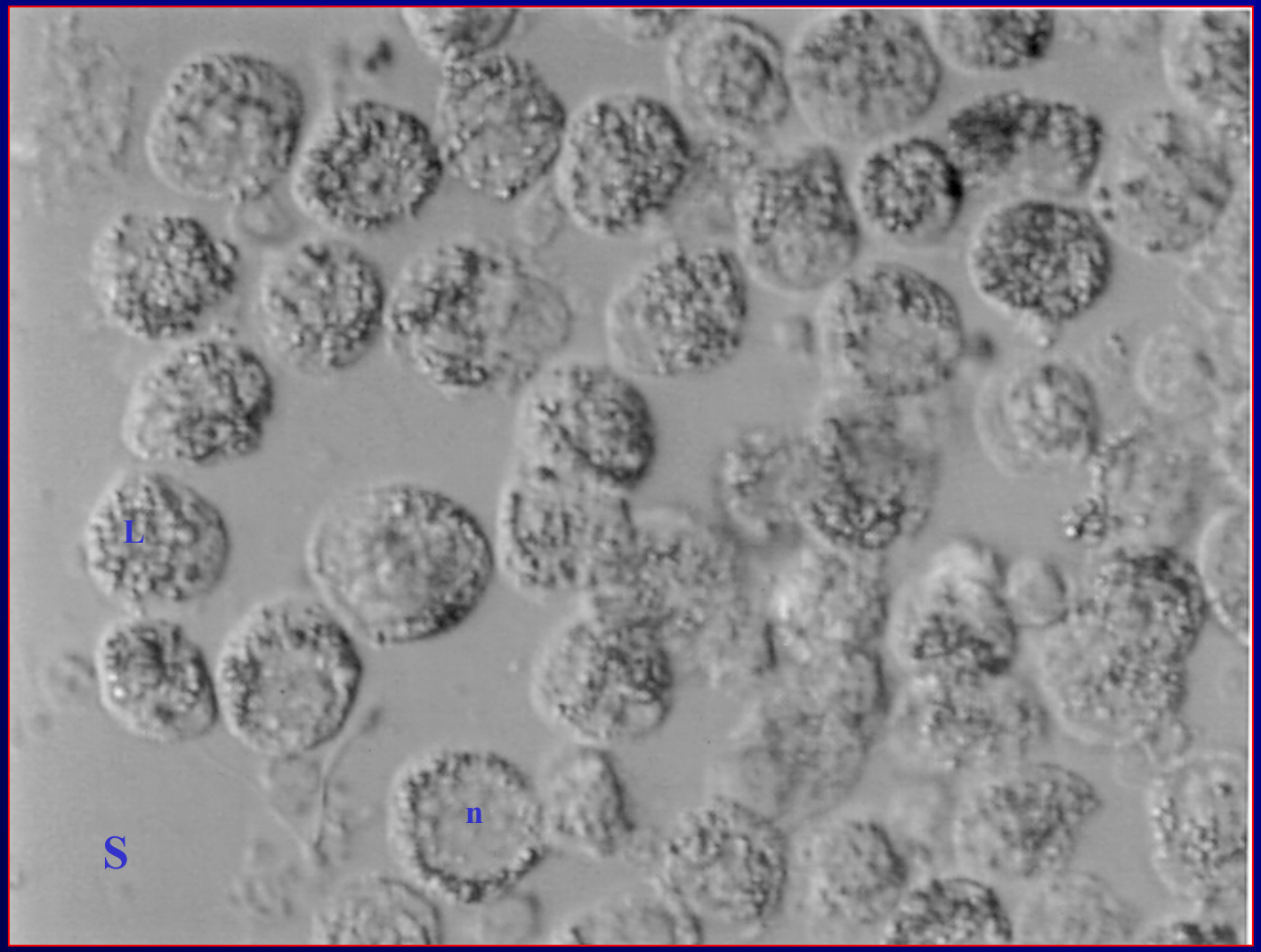

PTC


ST1
S
SG

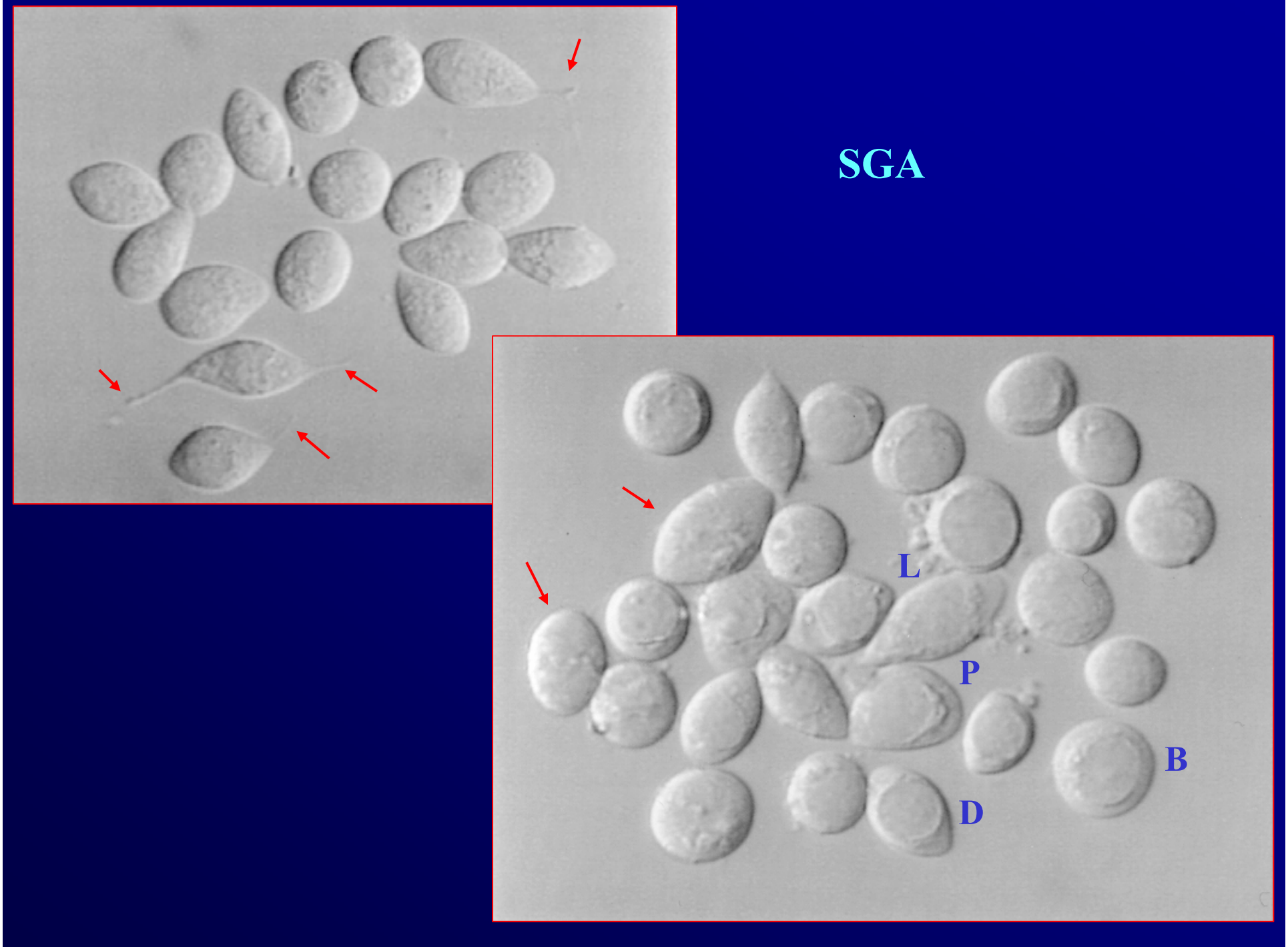



n


L


L
n
S

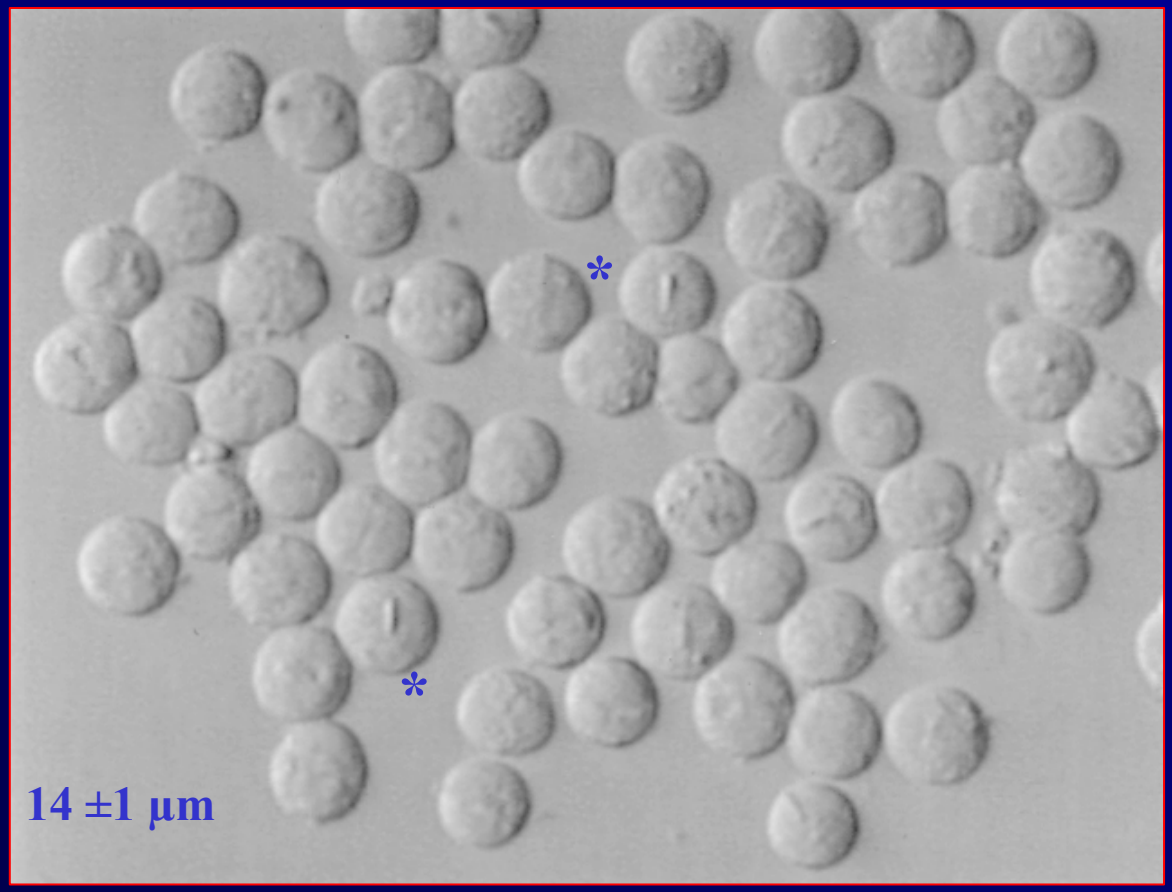
SGA

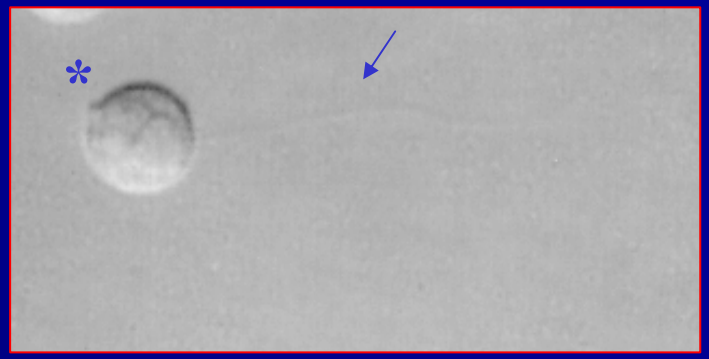


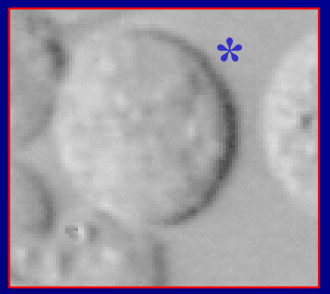
Meiosis I

2-4d

ST2

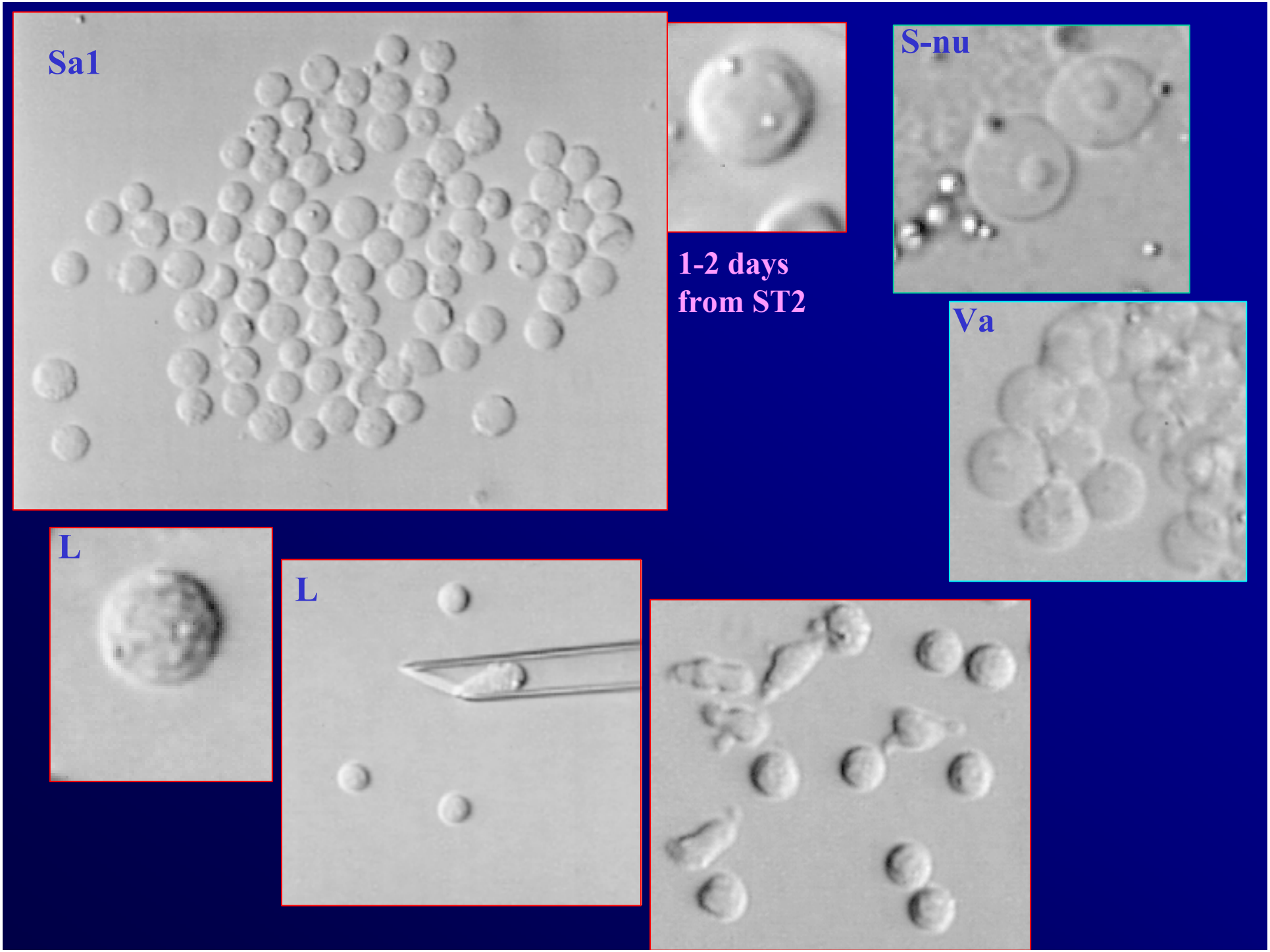


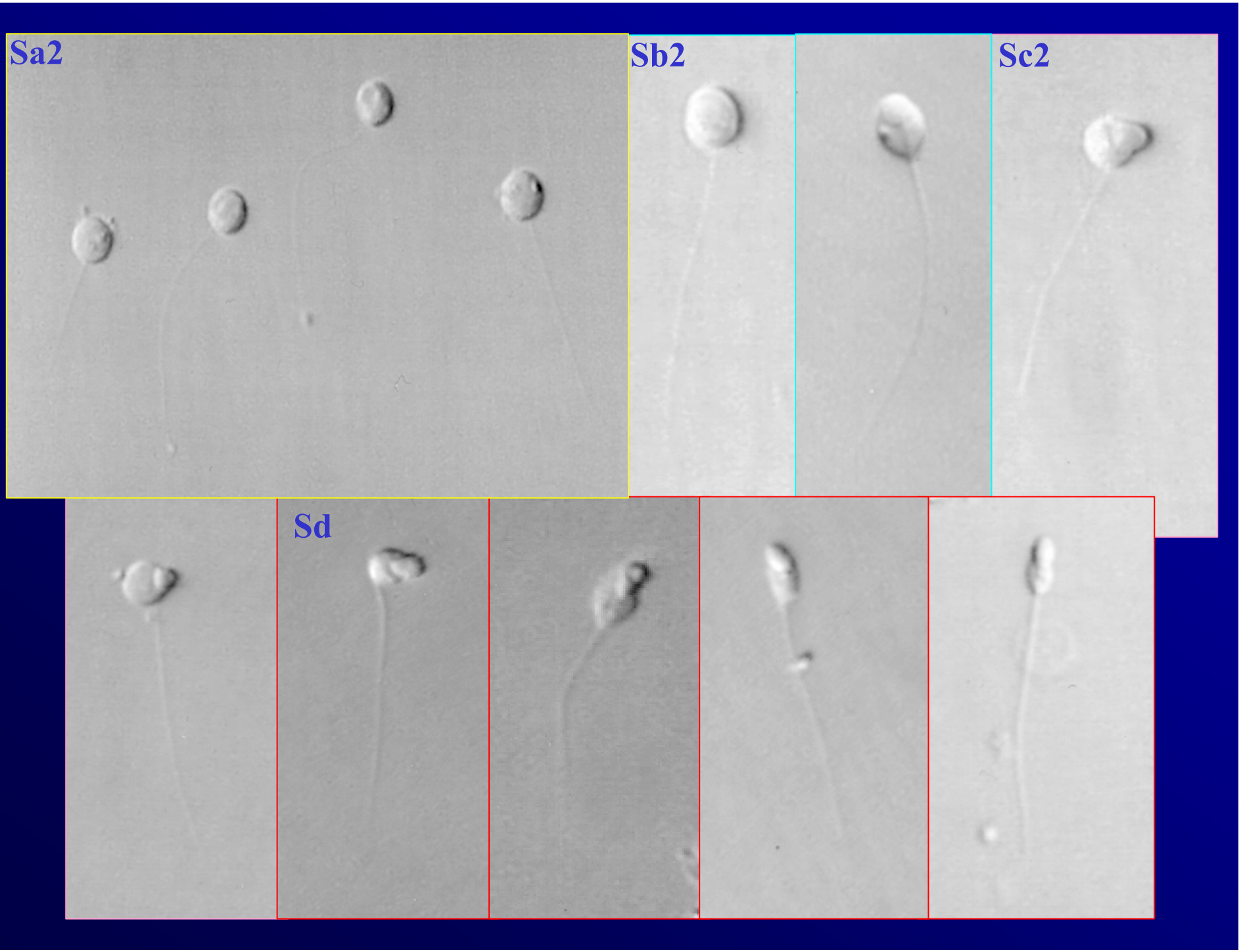


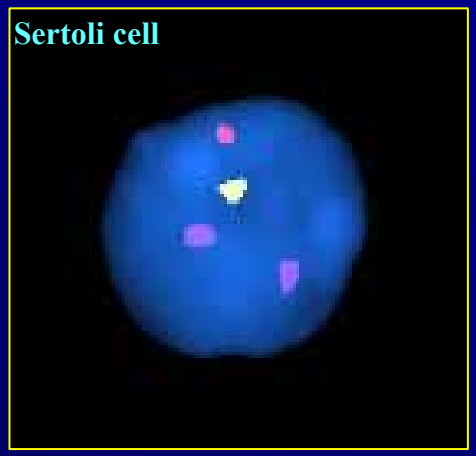


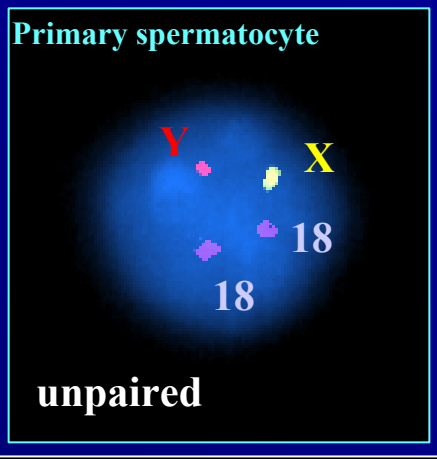
Meiosis II

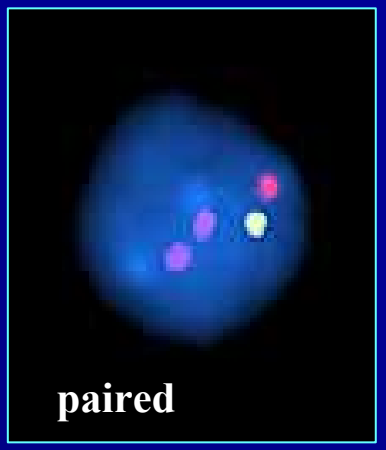
2-4 days from ST1

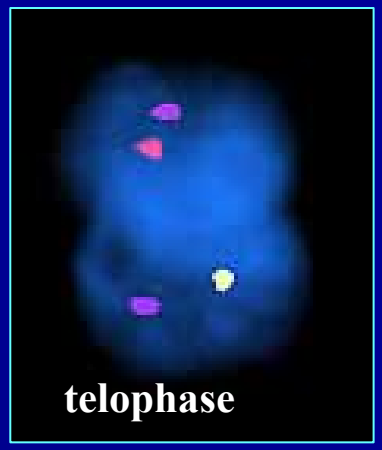

Sa1
1-2 days
from ST2
S-nu
Va
L
L

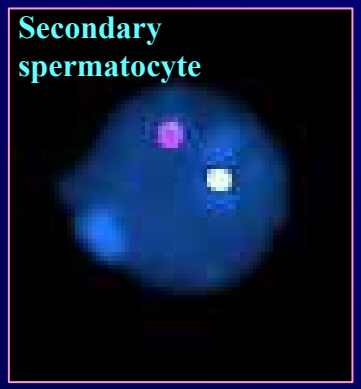

Sa2
Sb2
Sc2
Sd

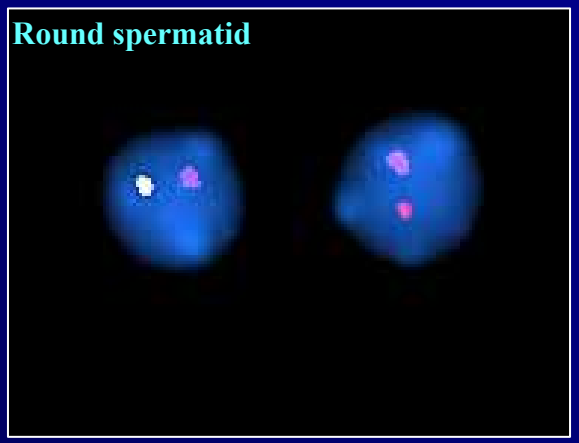


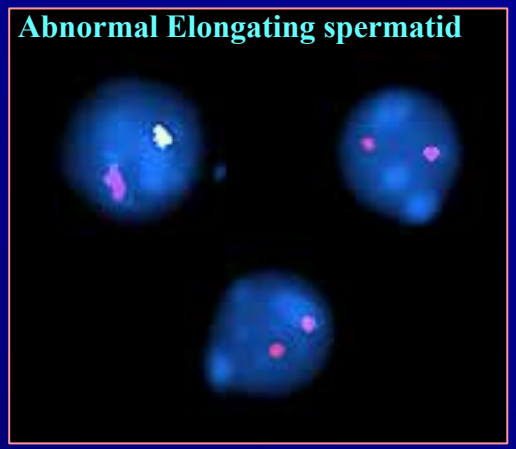


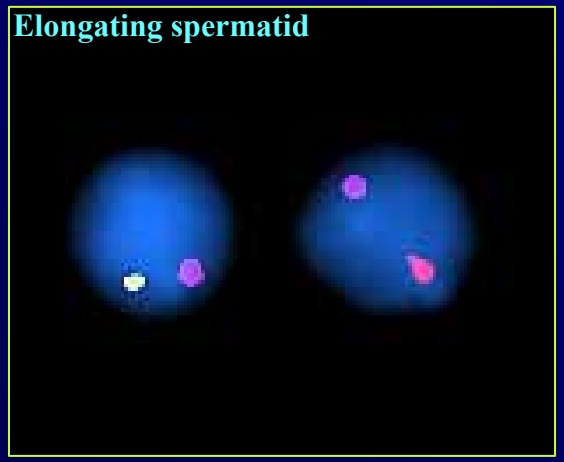


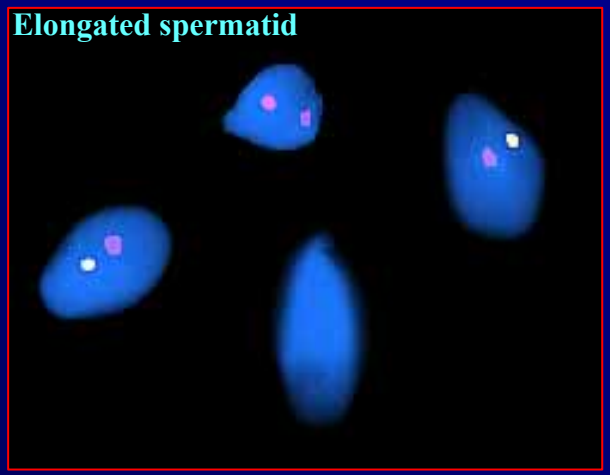












FISH analysis of the cells:

90% normal

Table I. In vitro differentiation of spermatids.

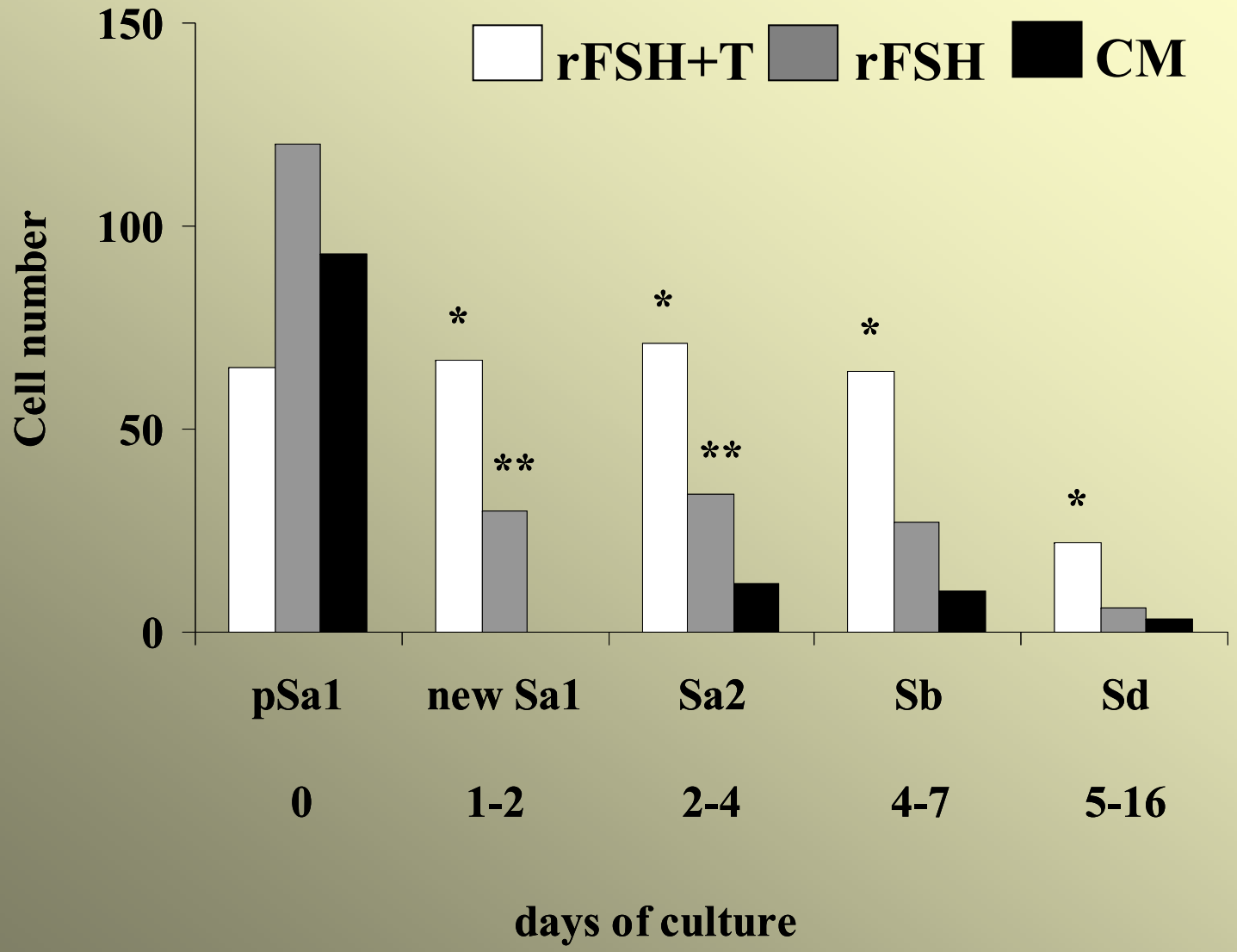
Cases			new Sa1	evolution of Sa1		evolution of Sa2		evolution of Sb	
Media	DGC	HGC		arrested	Sa2	arrested	Sb	arrested	Sd
CM	1000	93	0	81	12	2	10	7	3
CM+FSH	1000	120	30	116	34	7	27	21	6
CM+FSH+T	1000	65	67	61	71	7	64	42	22

HGC: haploid germ cells (Sa1) added to cultures.

Table II. Rates of spermatid in vitro differentiation.

Media	Total Sa1	Meiotic index	Spermatid evolution				
			Sa2	Sb		Sd	
	HGC+ new Sa1	new Sa1/ DGC	Sa2/ total Sa1	Sb/ total Sa1	Sb/Sa2	Sd/ total Sa1	Sd/Sb
CM	93	0/1000 (0)	12/93 (12.9)	10/93 (10.8)	10/12 (83.3)	3/93 (3.2)	3/10 (30)
CM+FSH	120	30/1000 (3)A	34/150 (22.7)B	27/150 (18)	27/34 (79.4)	6/150 (4)	6/27 (22.2)
CM+FSH+T	65	67/1000 (6.7)C	71/132 (53.8)C	64/132 (48.5)C	64/71 (90.1)	22/132 (16.7)C	22/64 (34.4)

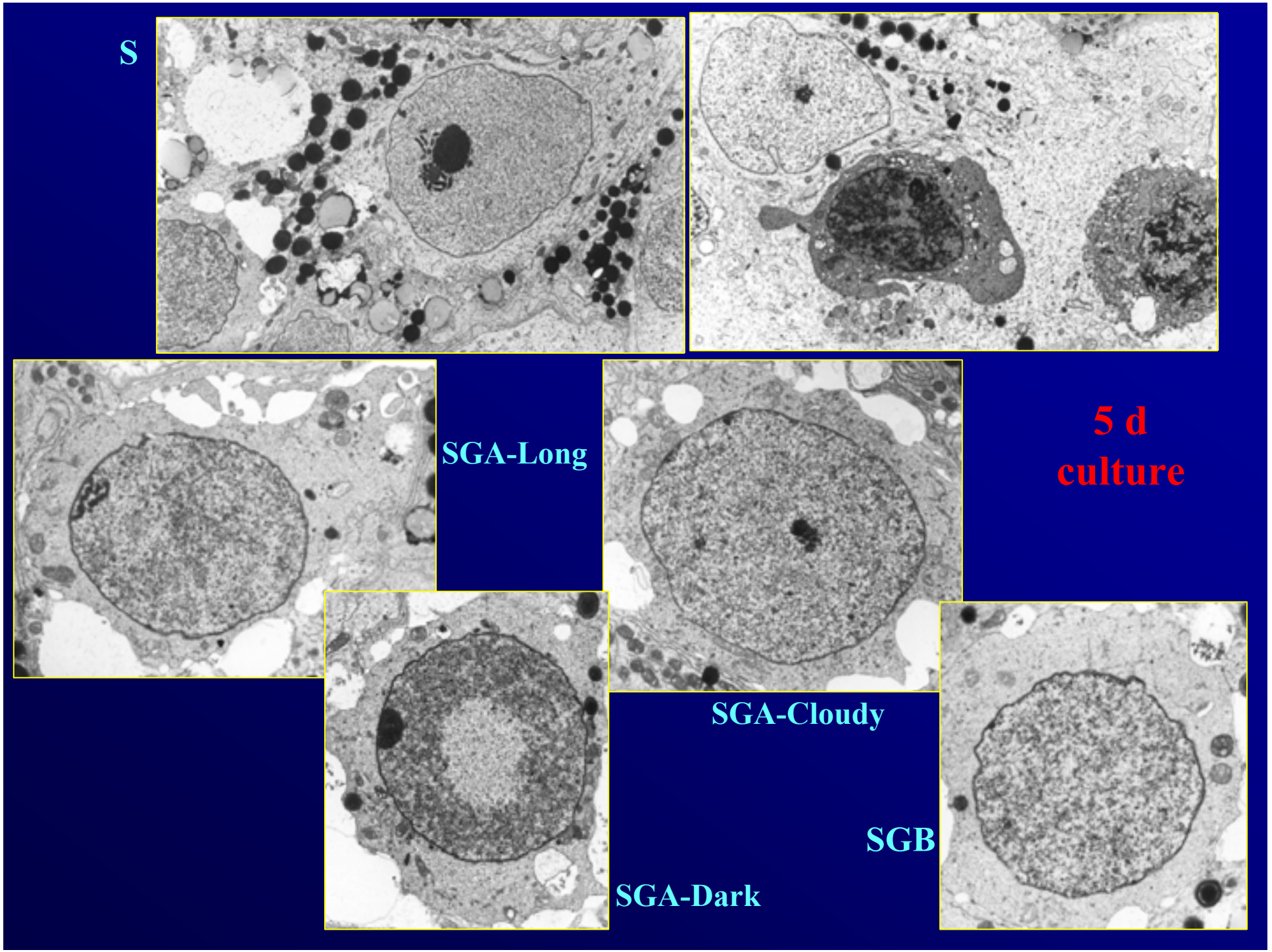
For each column:
(A) $P<0.01$ to CM; (B) $P<0.05$ to CM; (C) $P<0.01$ to CM and CM+FSH.


150
100
50
0
Cell number
rFSH+T
rFSH
CM
*
**
*
**
*
*
pSa1
new Sa1
Sa2
Sb
Sd
0
1-2
2-4
4-7
5-16
days of culture

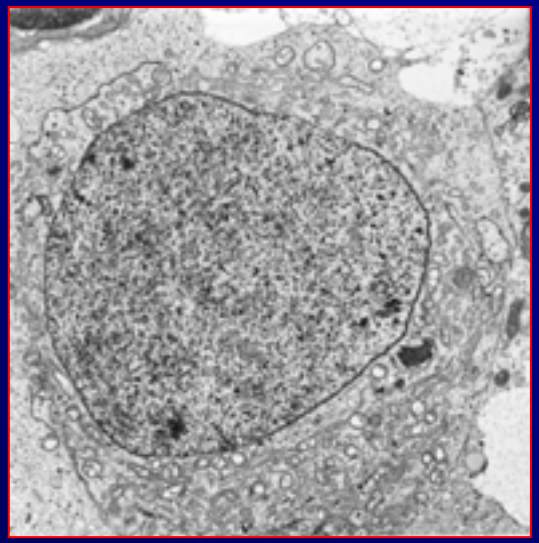
rFSH stimulated meiosis (new Sa1) and early spermatid maturation (Sa2: flagellum extrusion).

rFSH+T further stimulated meiosis and were active on all steps of spermiogenesis (Sa2, Sb and Sd).

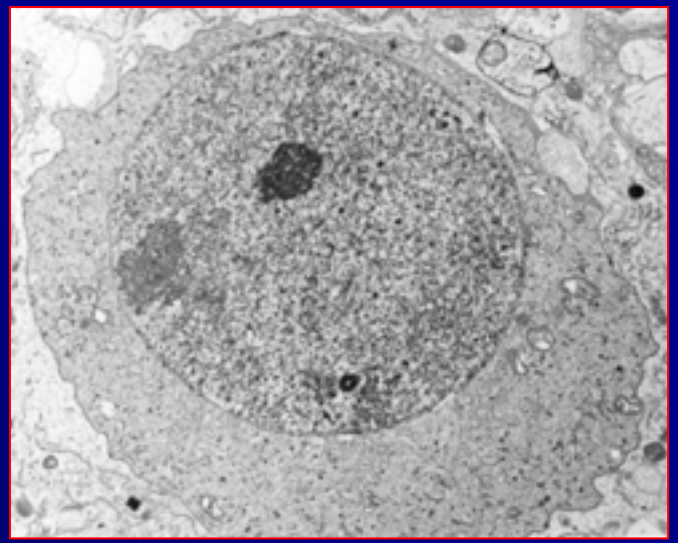
Spermatid differentiation needed a mean of 9 (5-16) days of culture.


S
SGA-Long
SGA-Cloudy
SGA-Dark
SGB
5 d
culture

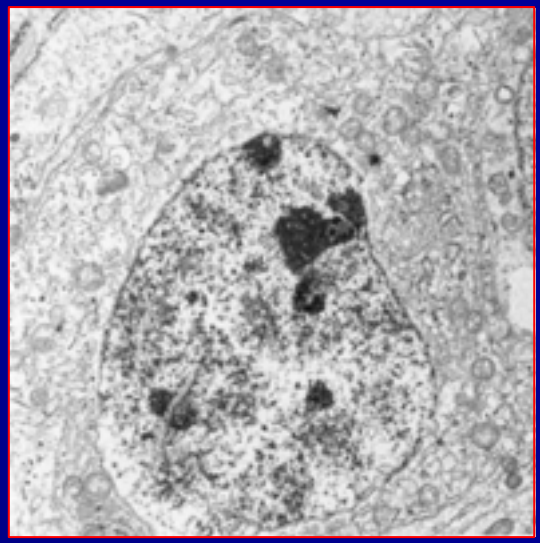
preLeptotene/Leptotene

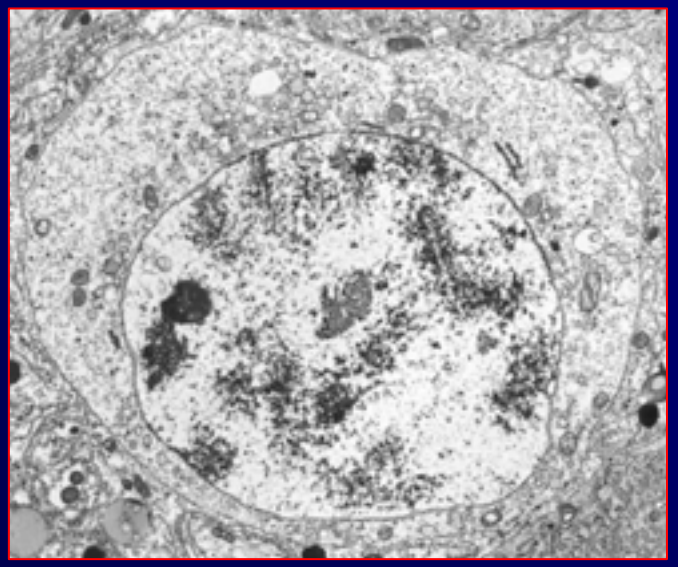


Early Zygotene

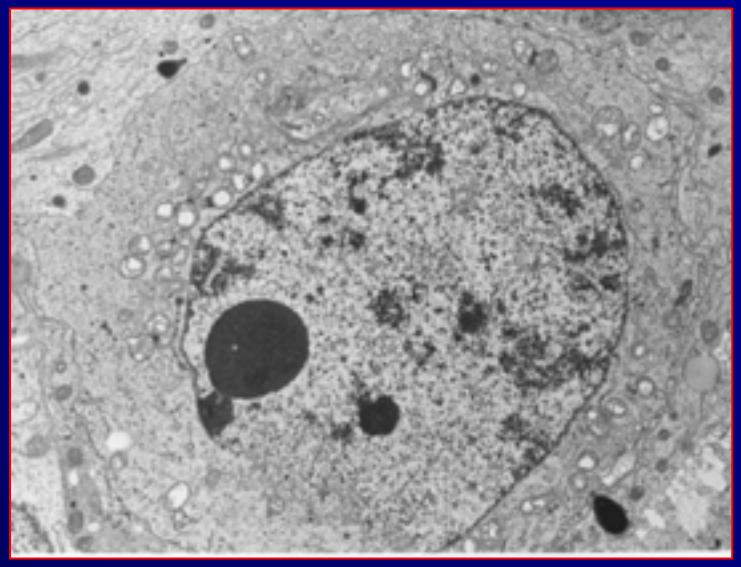


Late Zygotene





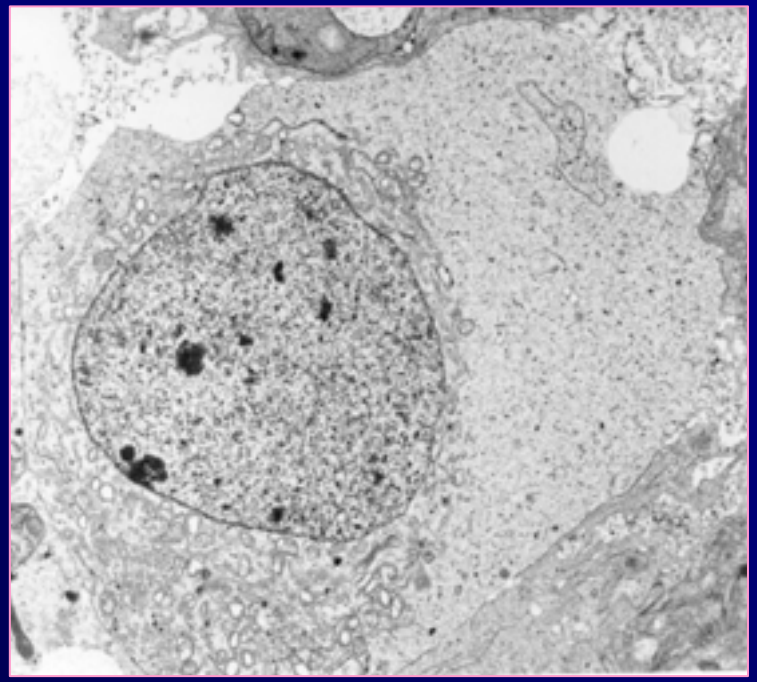
Early Pachytene



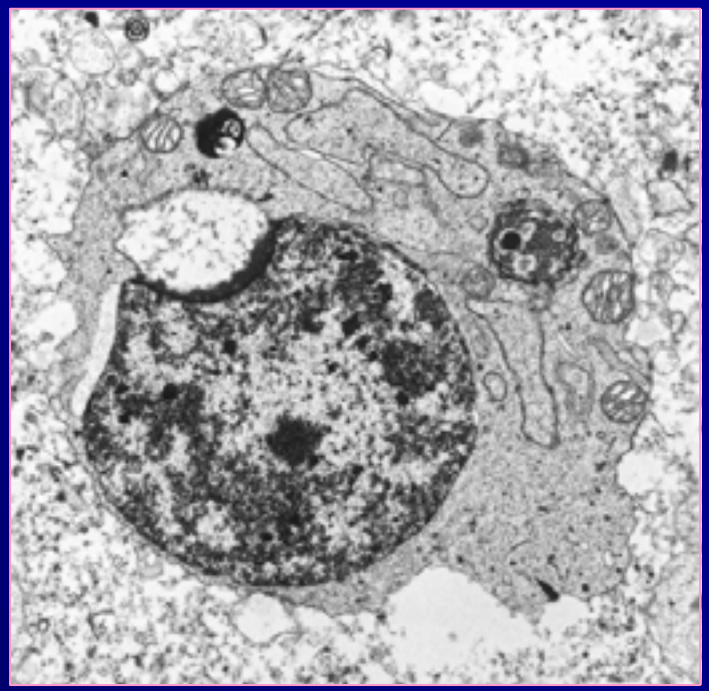
Late Pachytene

ST1

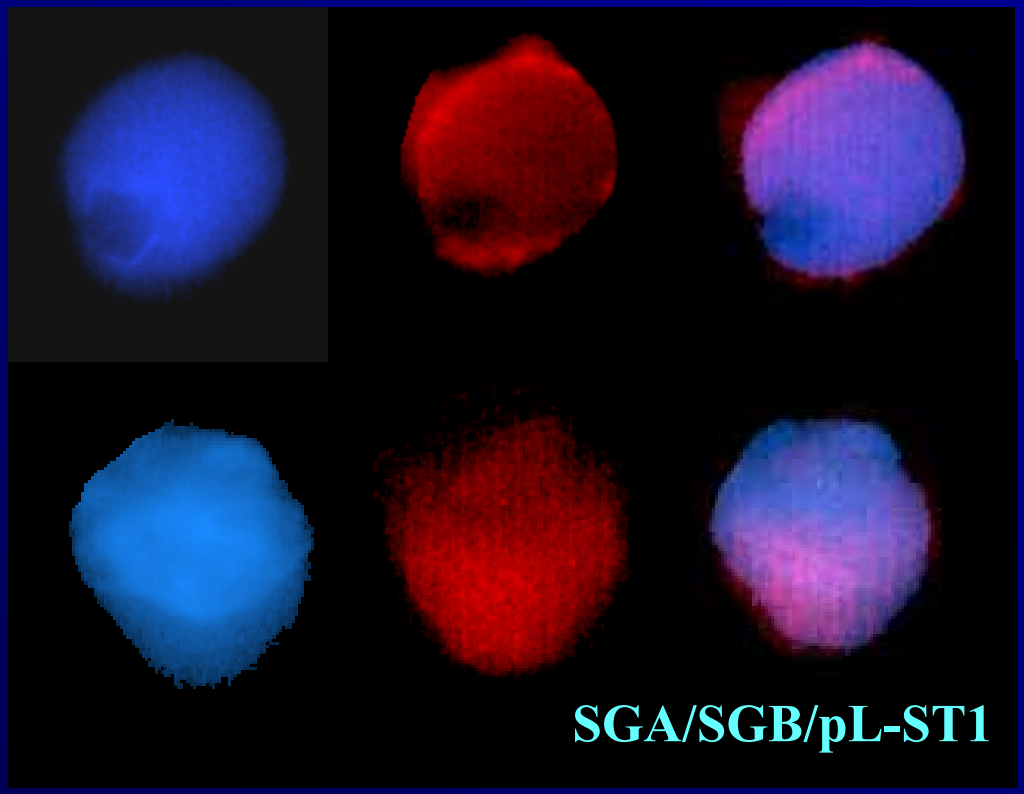
ST2



Sa1

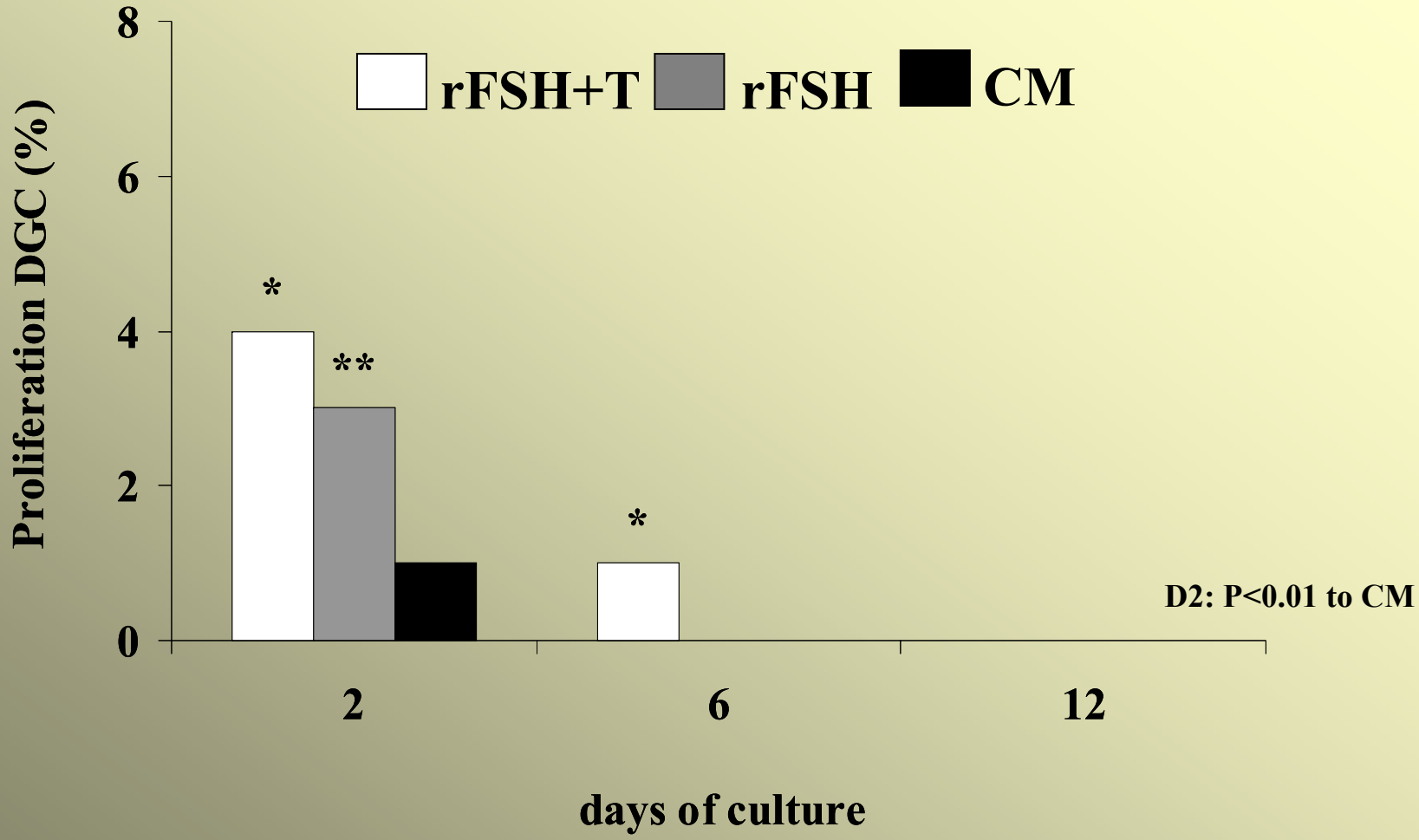


Cell junctions were partially reestablished between SC and DGC but not with Sa1.

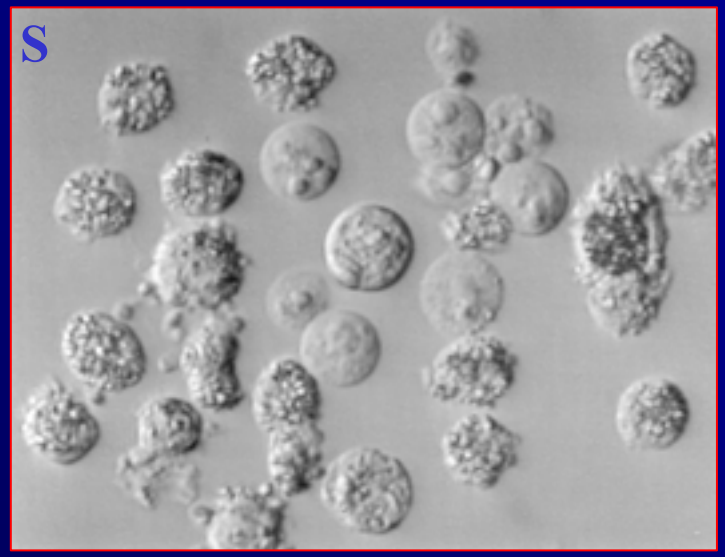


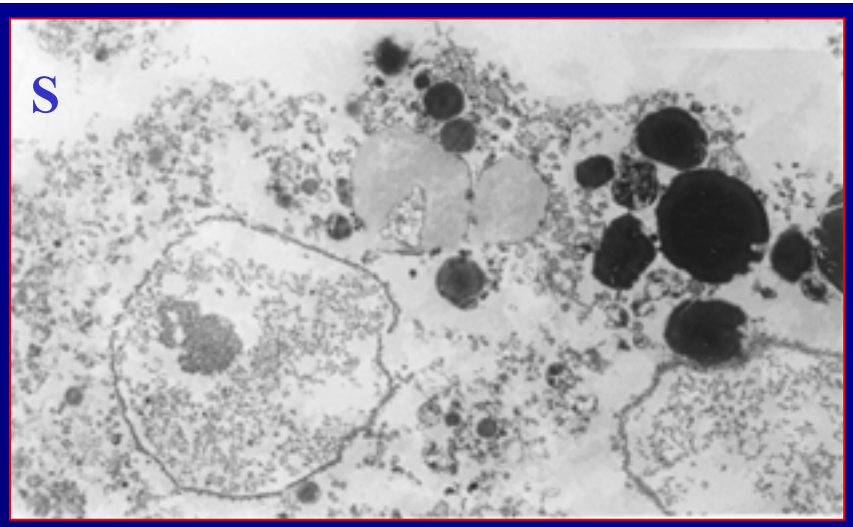
BrdU
incorporation – detection

DAPI - nu

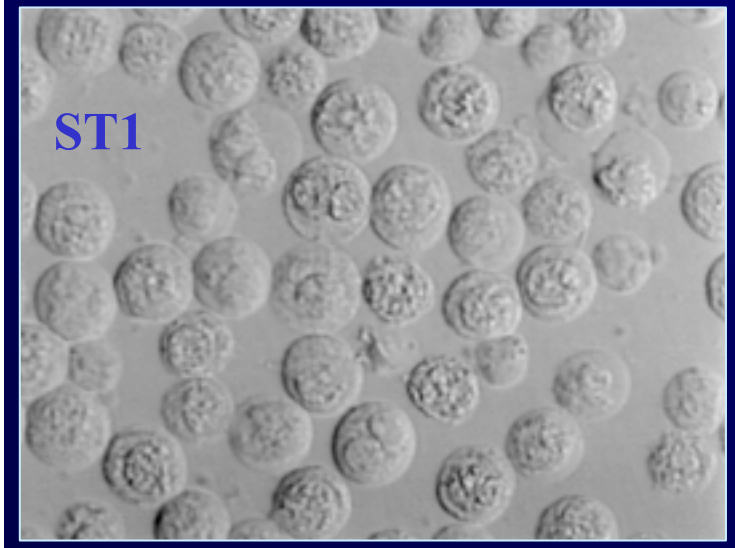


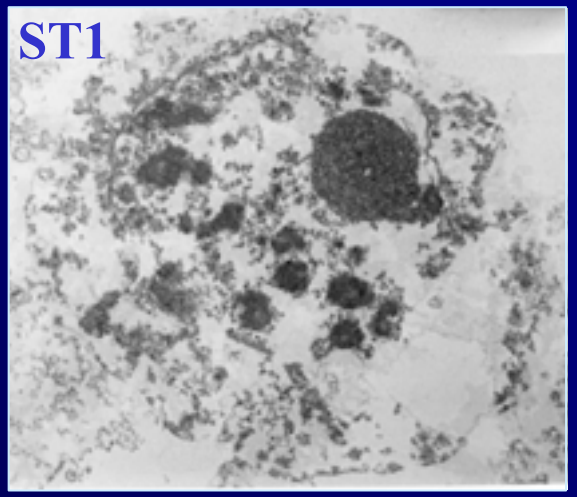
Germ cell proliferation appeared stimulated by both hormones during the first 2 days (4%), kept only under rFSH+T by day 6 (1%), and then stopped.

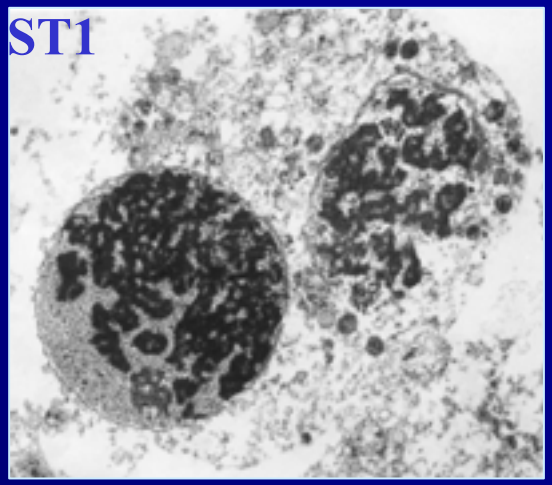


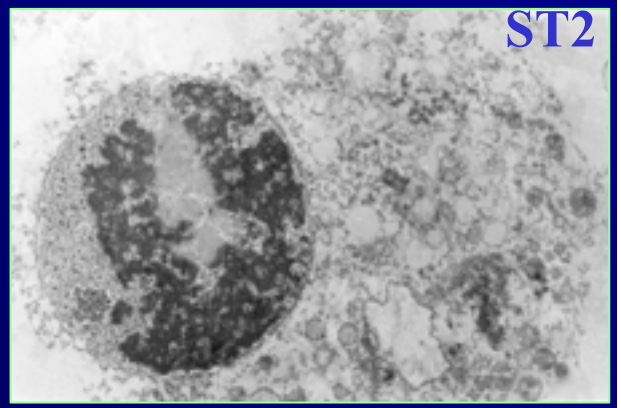


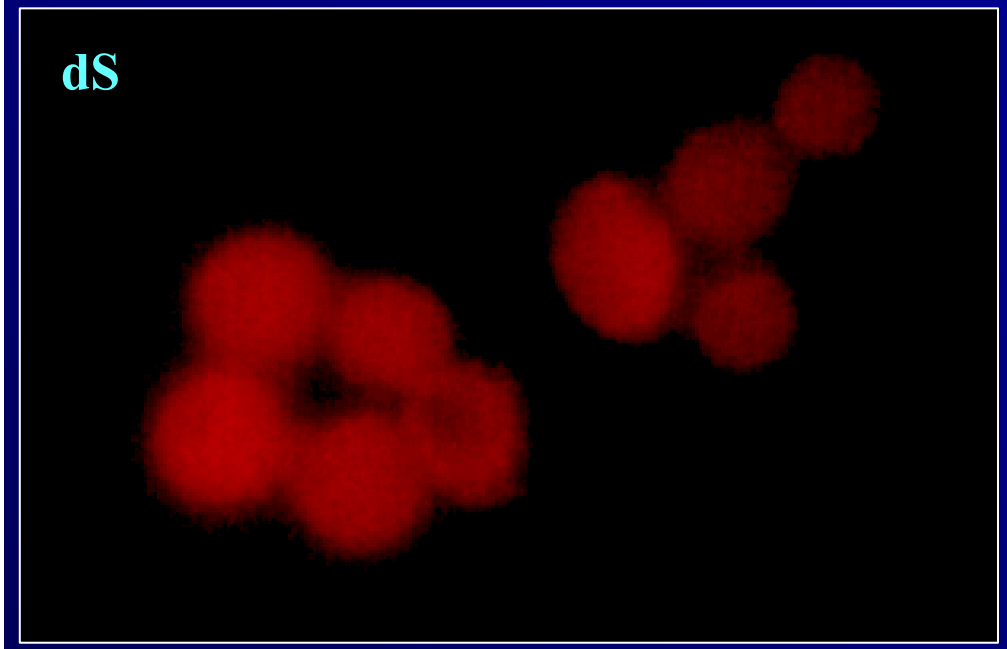
Apoptosis

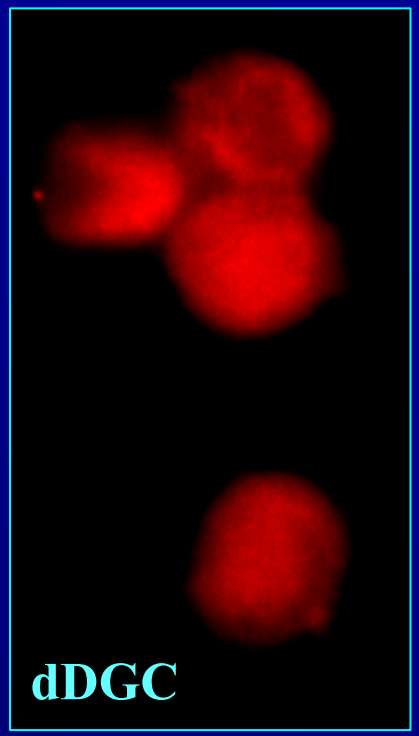


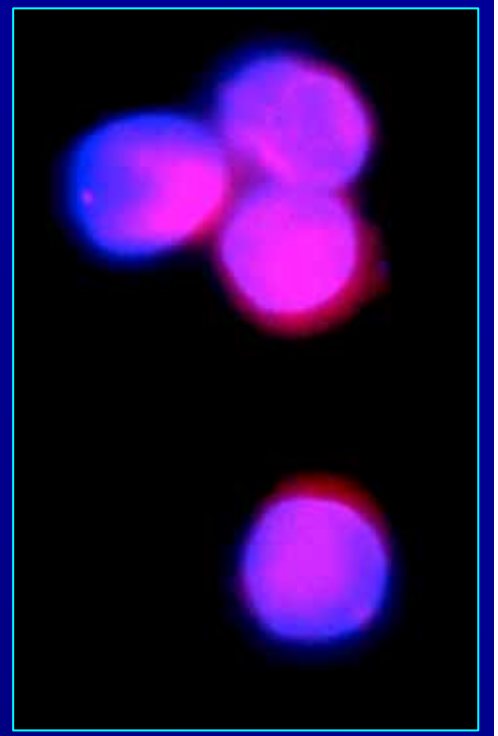






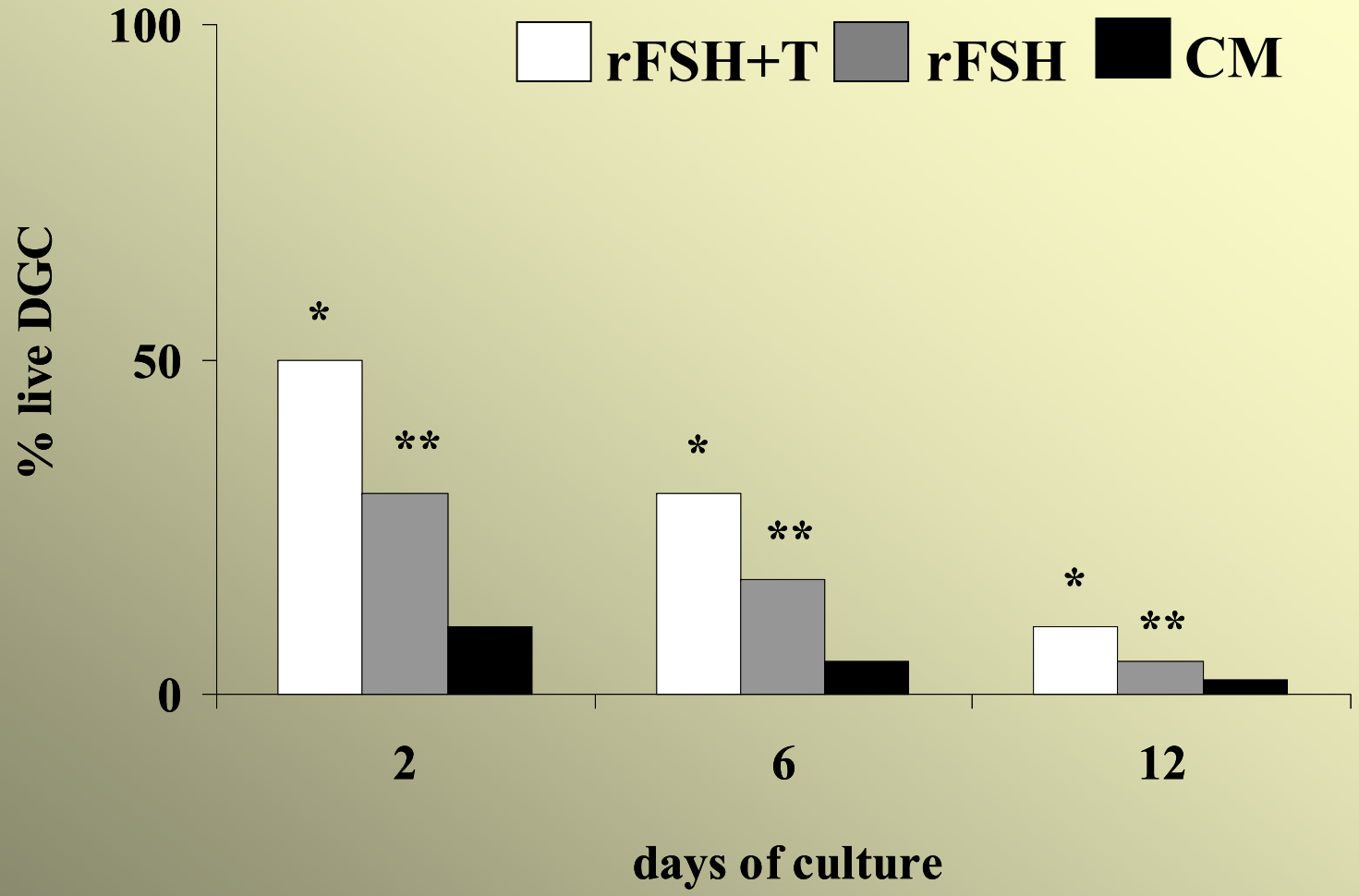




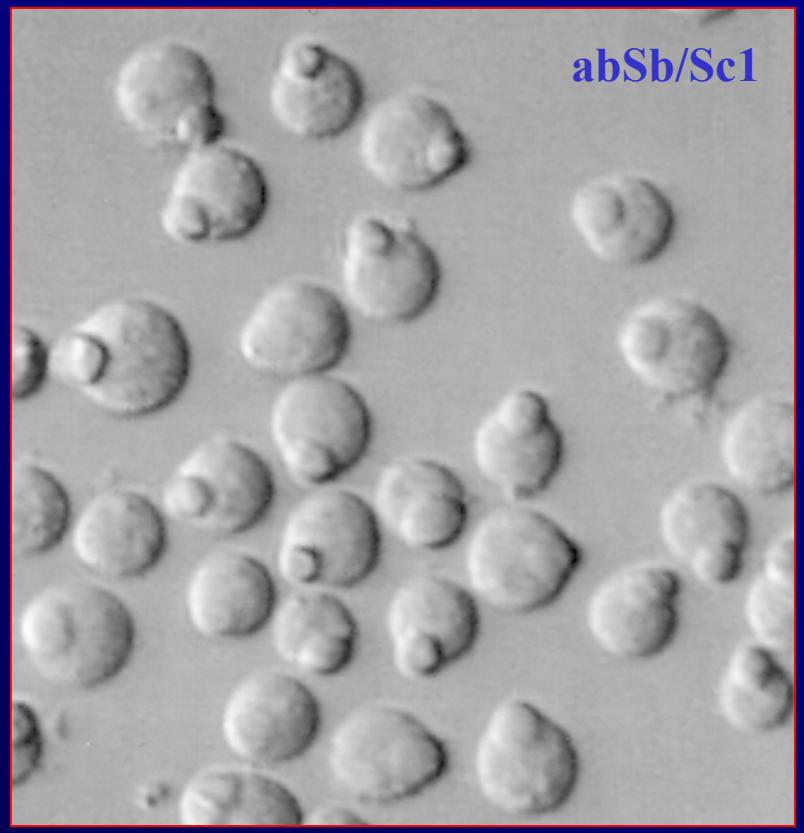


Caspase-3-like activity

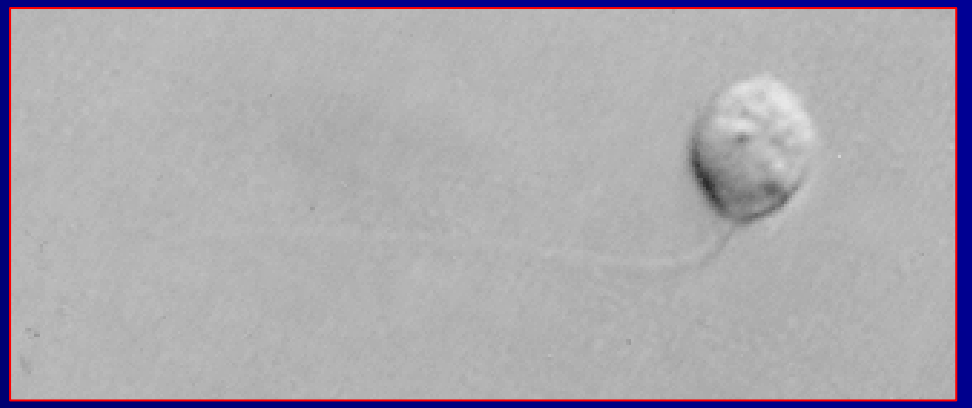
DAPI -nu

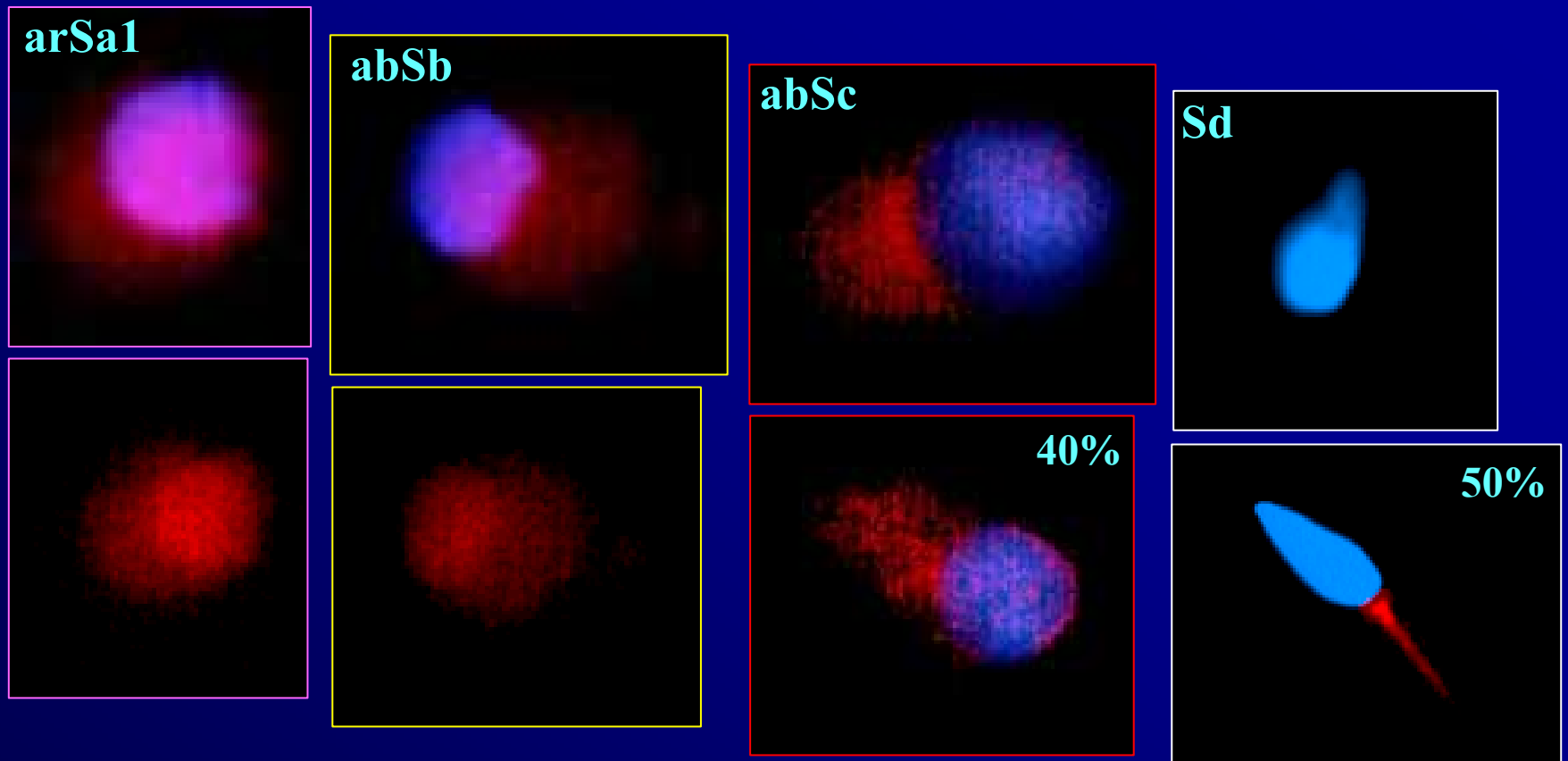


Apoptosis of SC and DGC was inhibited by rFSH and especially by rFSH+T, although degeneration of DGC continued at a high rate.


abSb/Sc1


abSb/Sc2

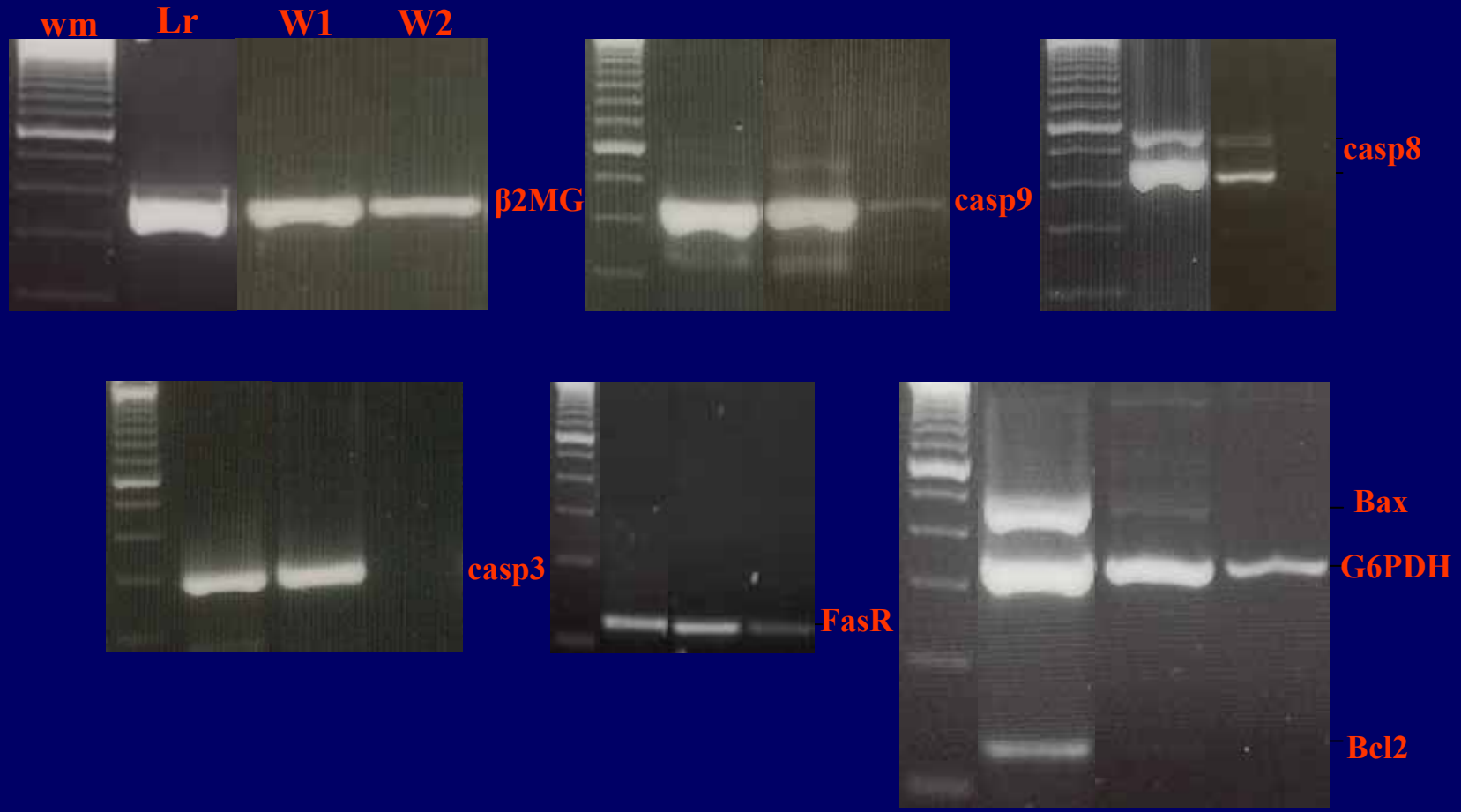




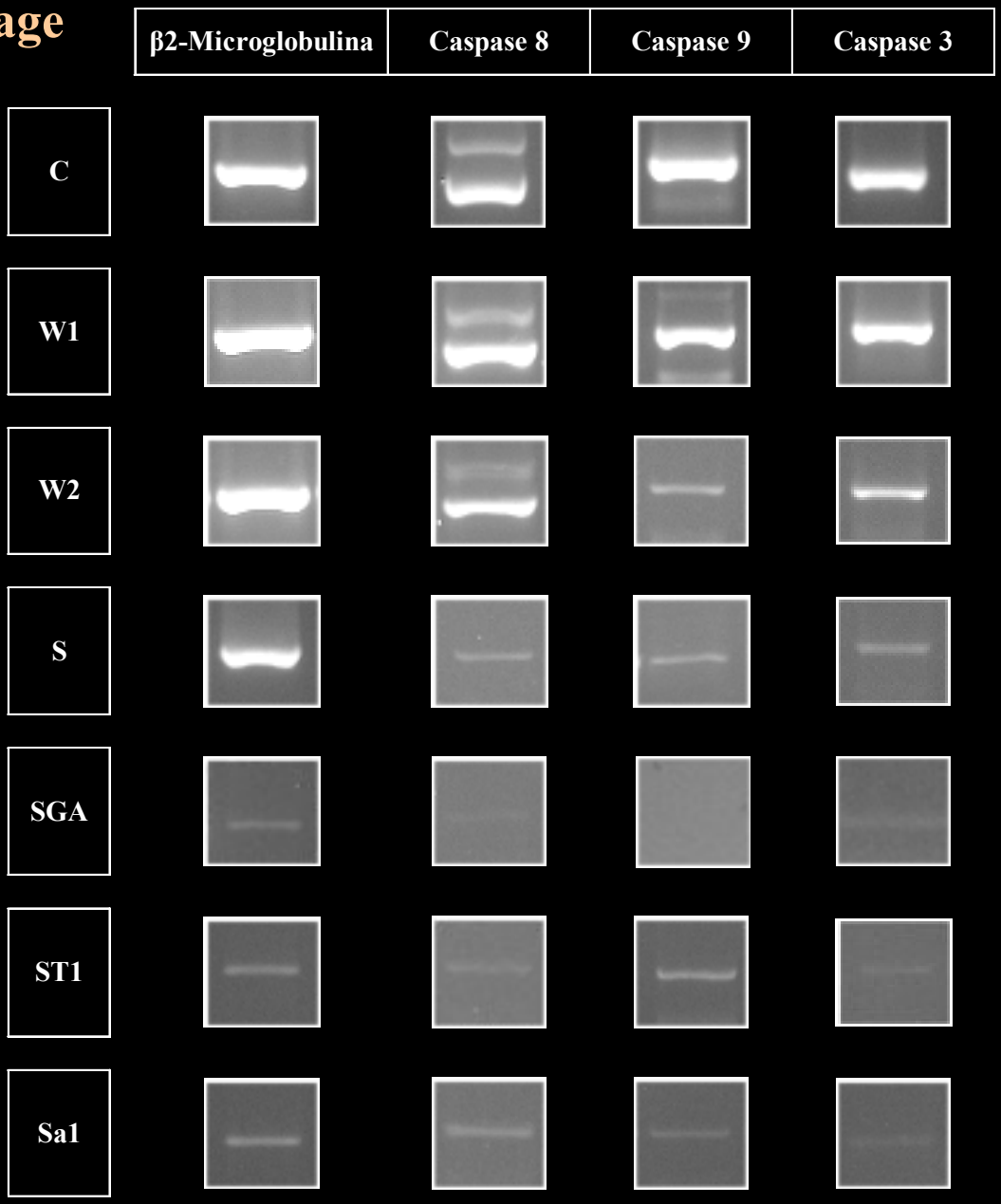
Caspase-3-like activity

DAPI -nu

Apoptosis markers



apoptosis
caspase activity per cell stage



FINAL CONCLUSIONS

The present data suggest that long term in vitro cocultures of the normal human seminiferous epithelium sustain meiosis (7%) and full germ cell differentiation (17%), at a physiological pace (2-3 weeks).

However, more complex media should be evaluated as the rates of cell proliferation (4%) and meiosis completion (7%) were limited by high levels of DGC apoptosis (70% in the 1st week) and detachment of spermatids from SC intercellular connections.