Recent trends in Reproductive Biotechnology in Livestock

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REPRODUCTIVE TECHNOLOGIES

Ovum Pick Up (OPU)

Preantral follicle Technology

In vitro embryo production / cloning

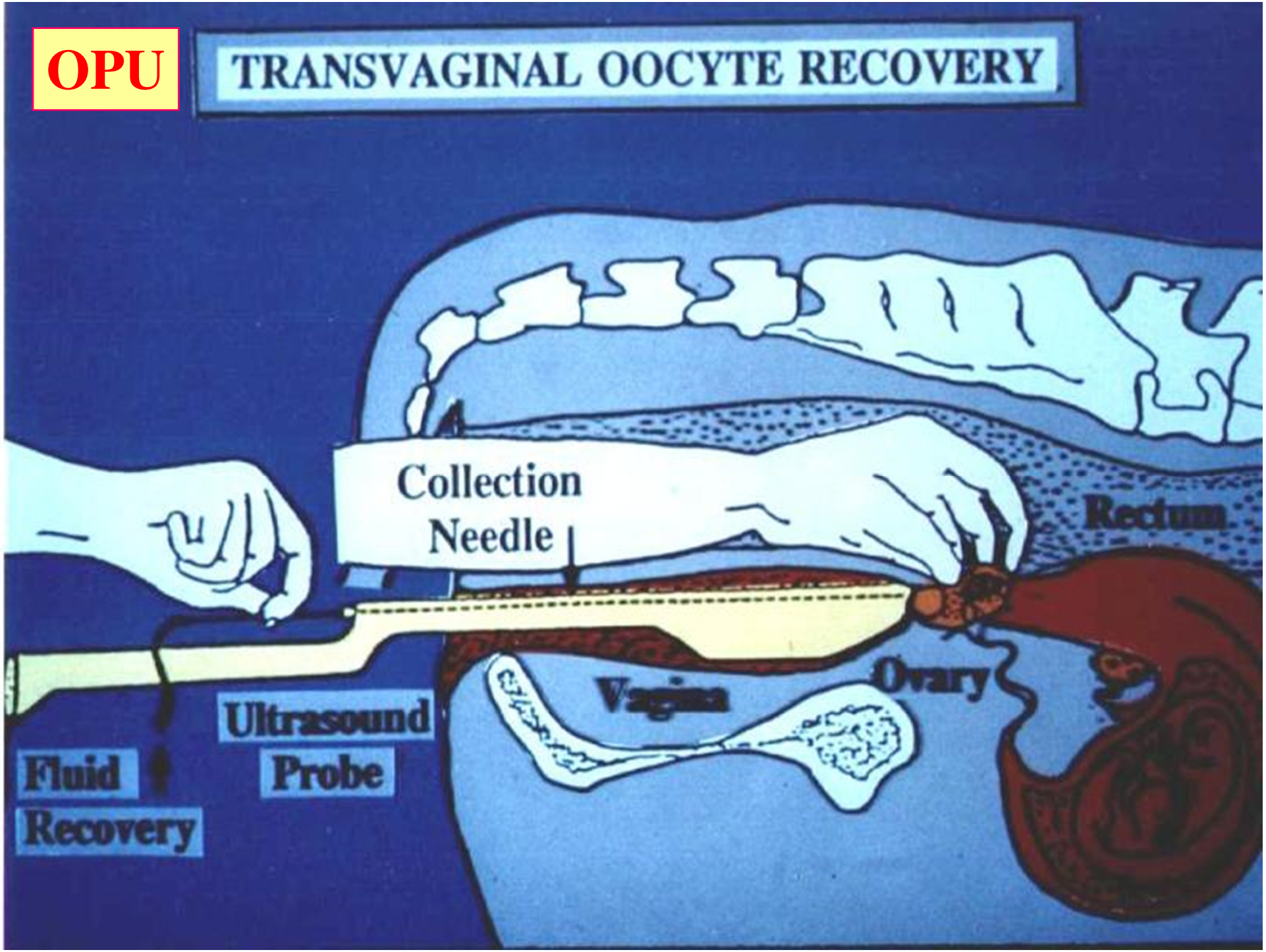
Sperm / Embryo Sexing

Transgenic Animal production / cloning

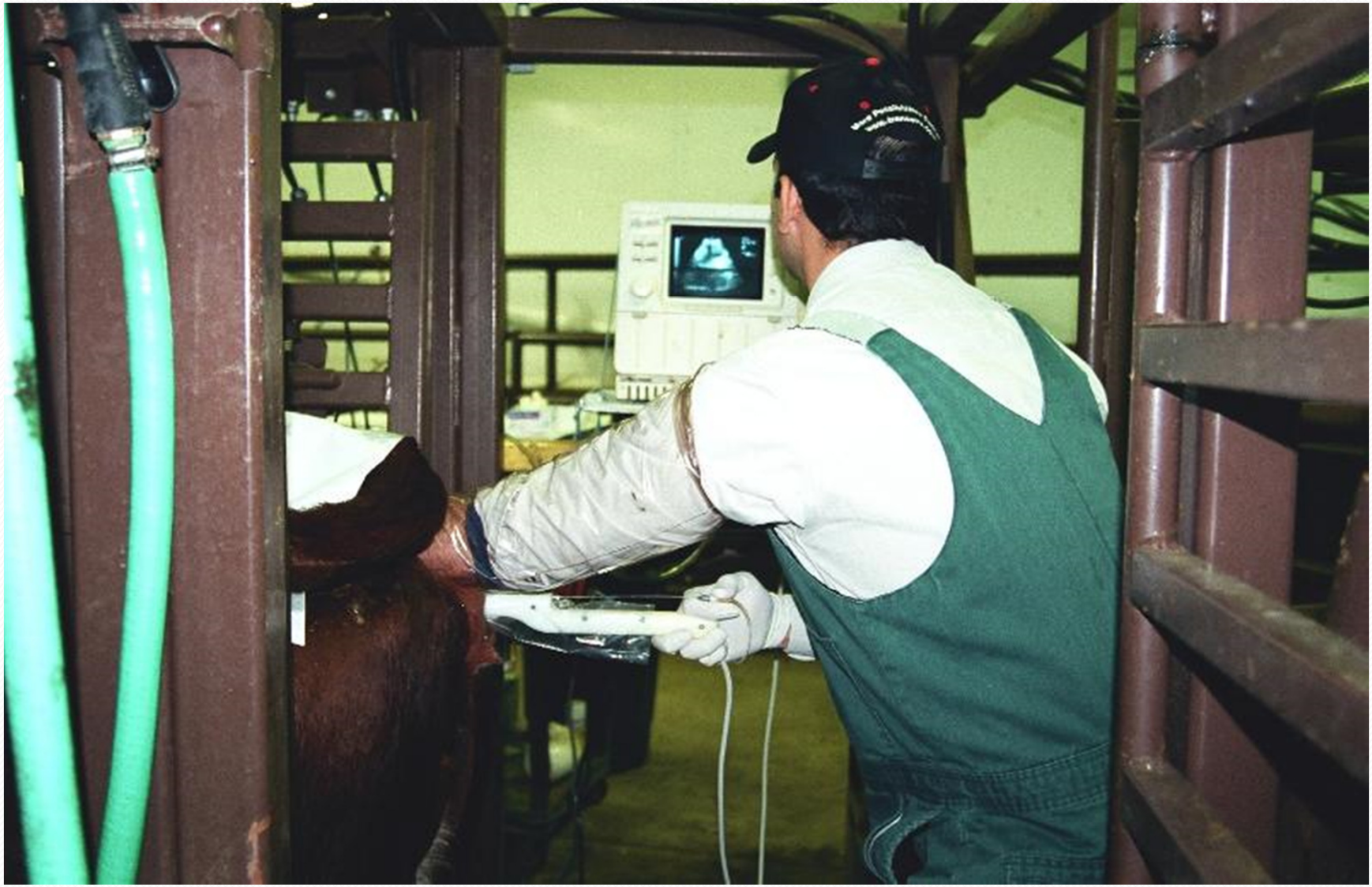
Stem cell Technology

OPU

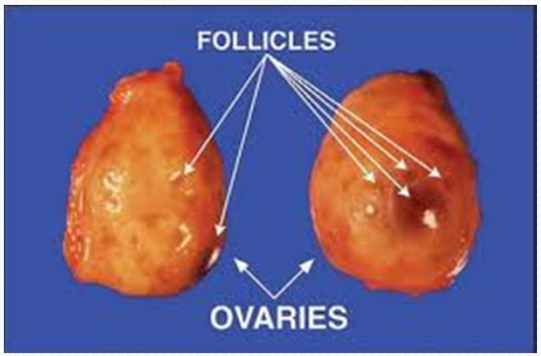
- ultrasound scanner (Aloka SSD 500, Japan)
- 5 MHz convex array transducer (Aloka, Japan)
- ovum aspiration needle (17 G, 50 cm)
- connected to an aspiration line made of silicon tubing (Cook, Australia)
- vacuum pump –
  pressure (80-120 mm Hg) / flow rate (20 ml /mn)


OPU
TRANSVAGINAL OOCYTE RECOVERY
Collection Needle
Rectum
Vagina
Ovary
Ultrasound Probe
Fluid Recovery

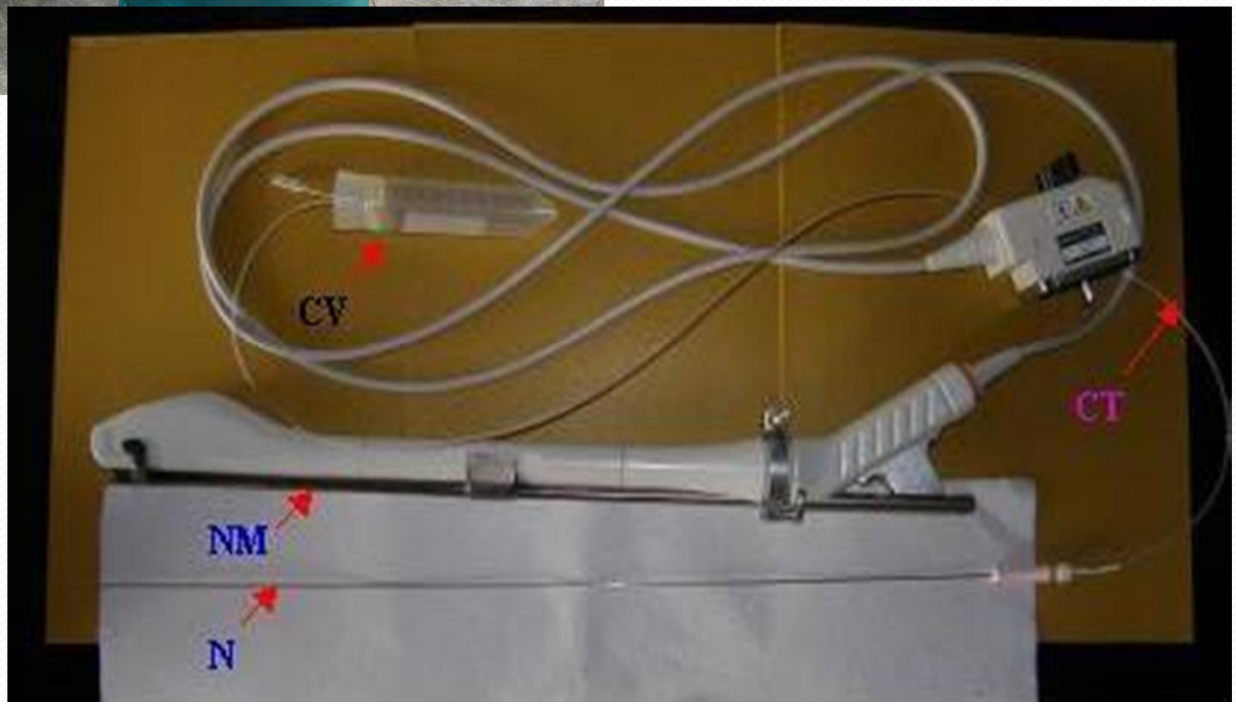
Transvaginal Ultrasound Guided Follicle Aspiration (OPU)






FOLLICLES
OVARIES

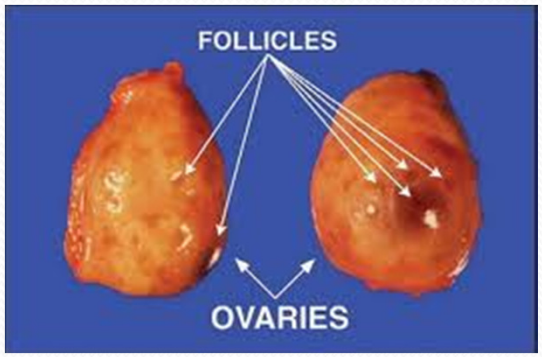



CV
CT
NM
N


RIC5-9-D/GYN
MI 0.8
Advanced Fertility Center
4.2cm / 1.2 / 25Hz
TIs 0.1
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IVF Egg Retrieval Procedure

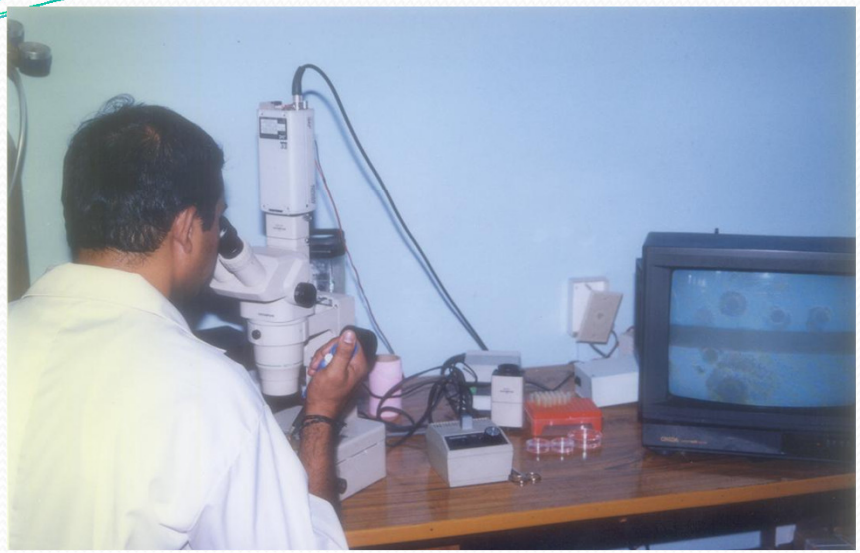
In vitro embryo production

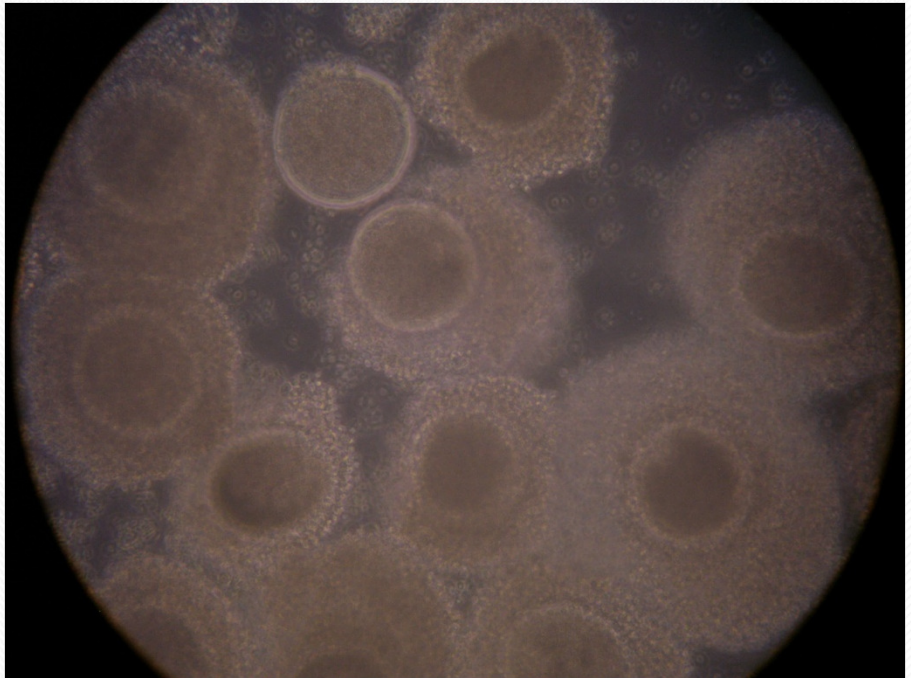
- 24 hr culture of oocytes in CO_2 Incubator
- In vitro sperm capacitation and IVF
- Embryo culture

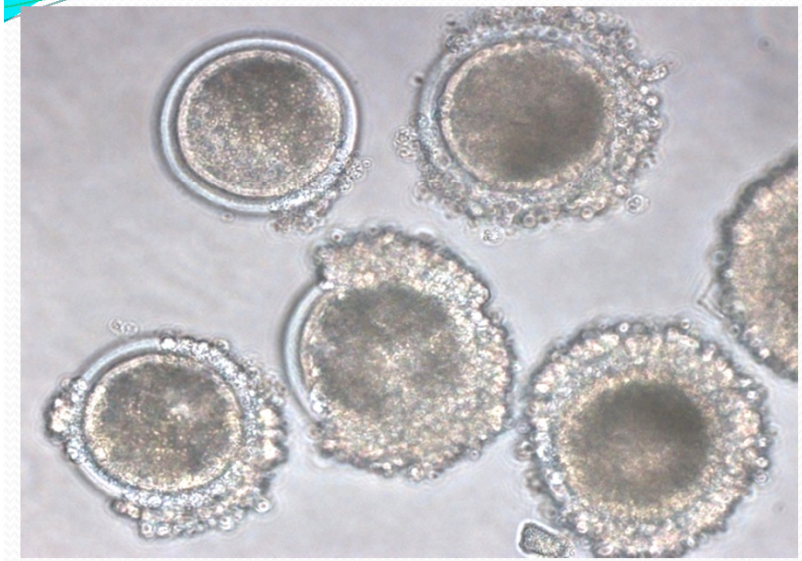


Important components in the In vitro culture medium

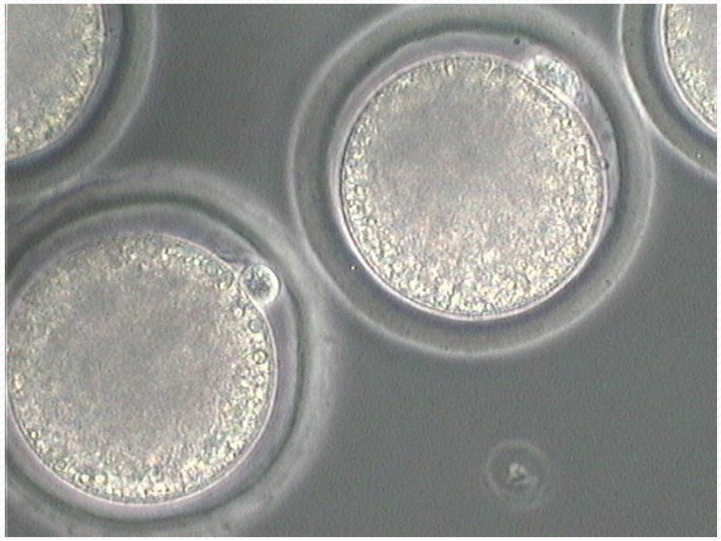
- Base medium like TCM-199, MEM, Waymouth, Hams etc. (consists of a.a., salts, Vit.,Glucose etc .)
- Serum (Fetal bovine serum / steer serum)
- Antibiotic
- Sodium pyruvate
- Glutamine
- Hypoxanthine



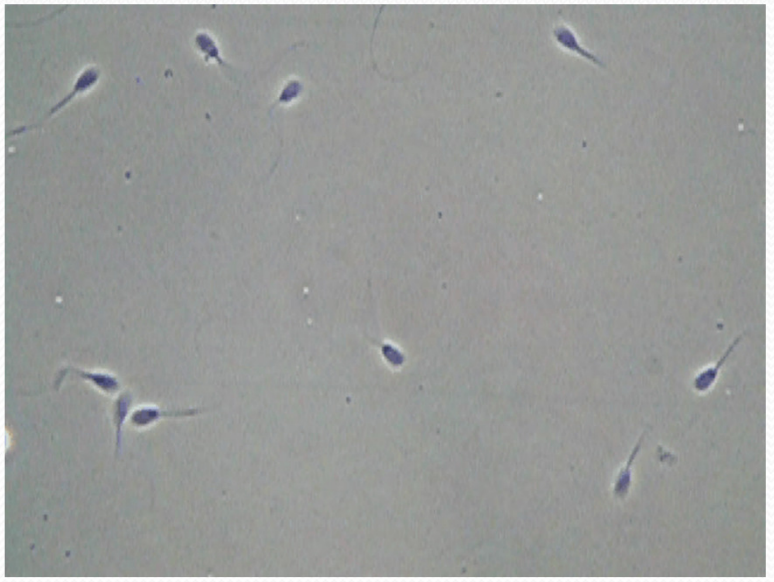


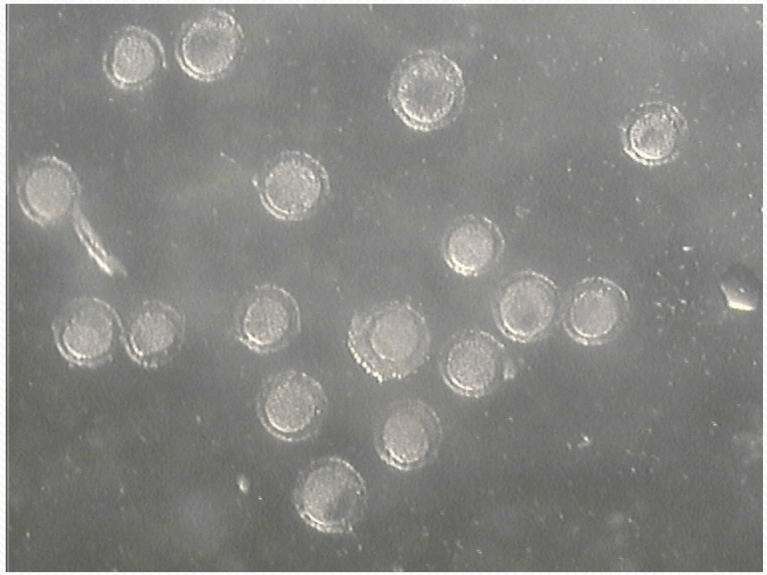


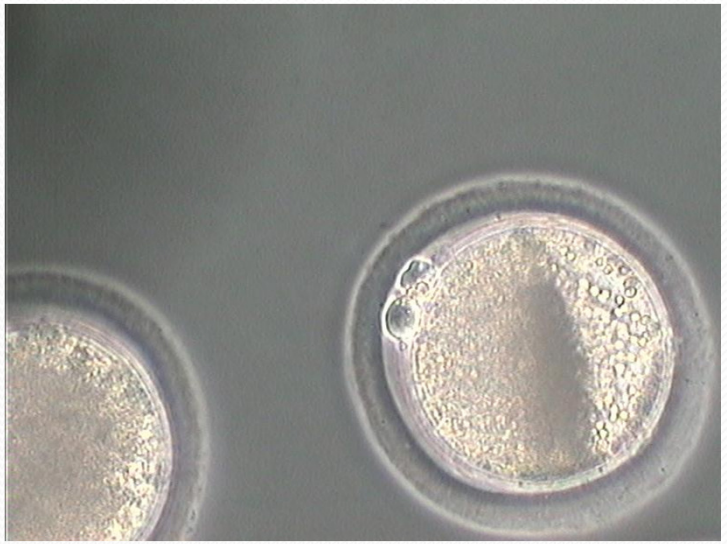




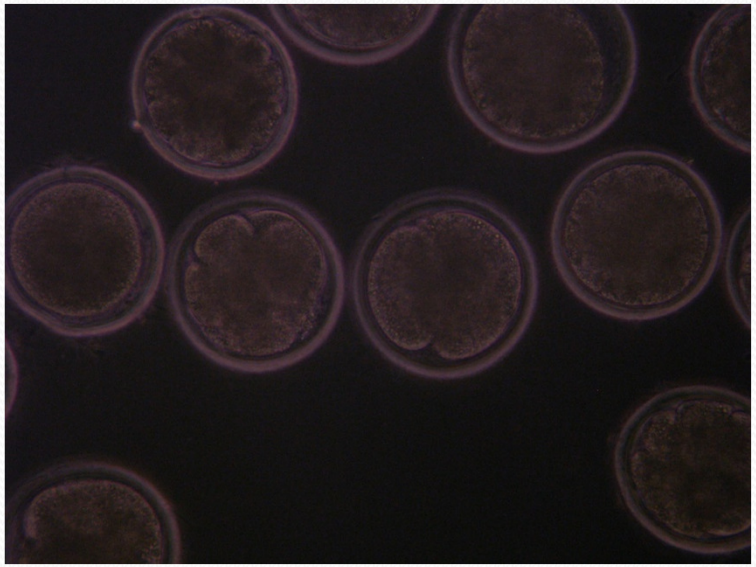


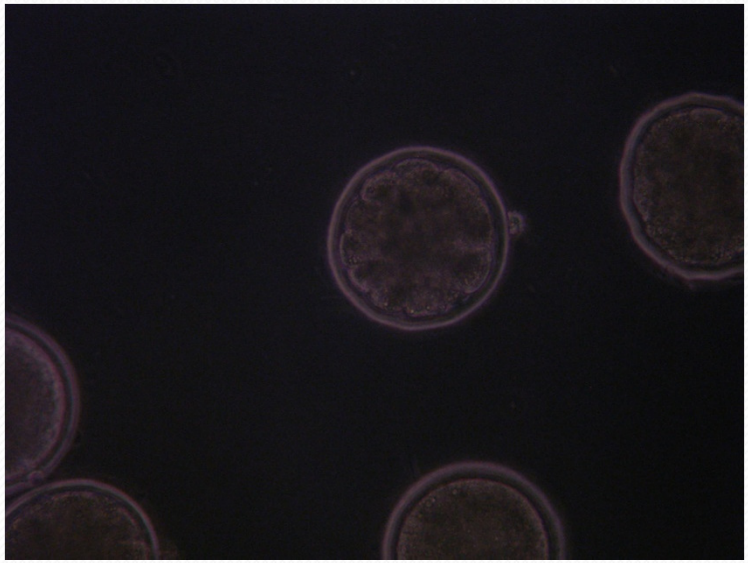








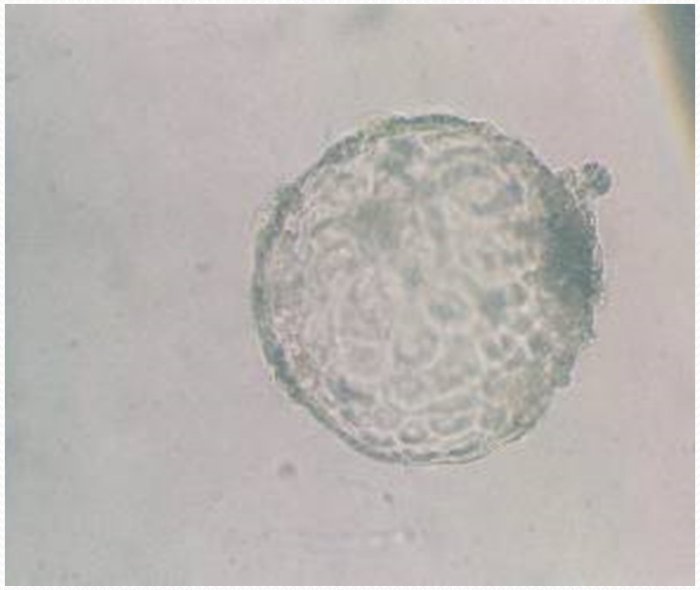




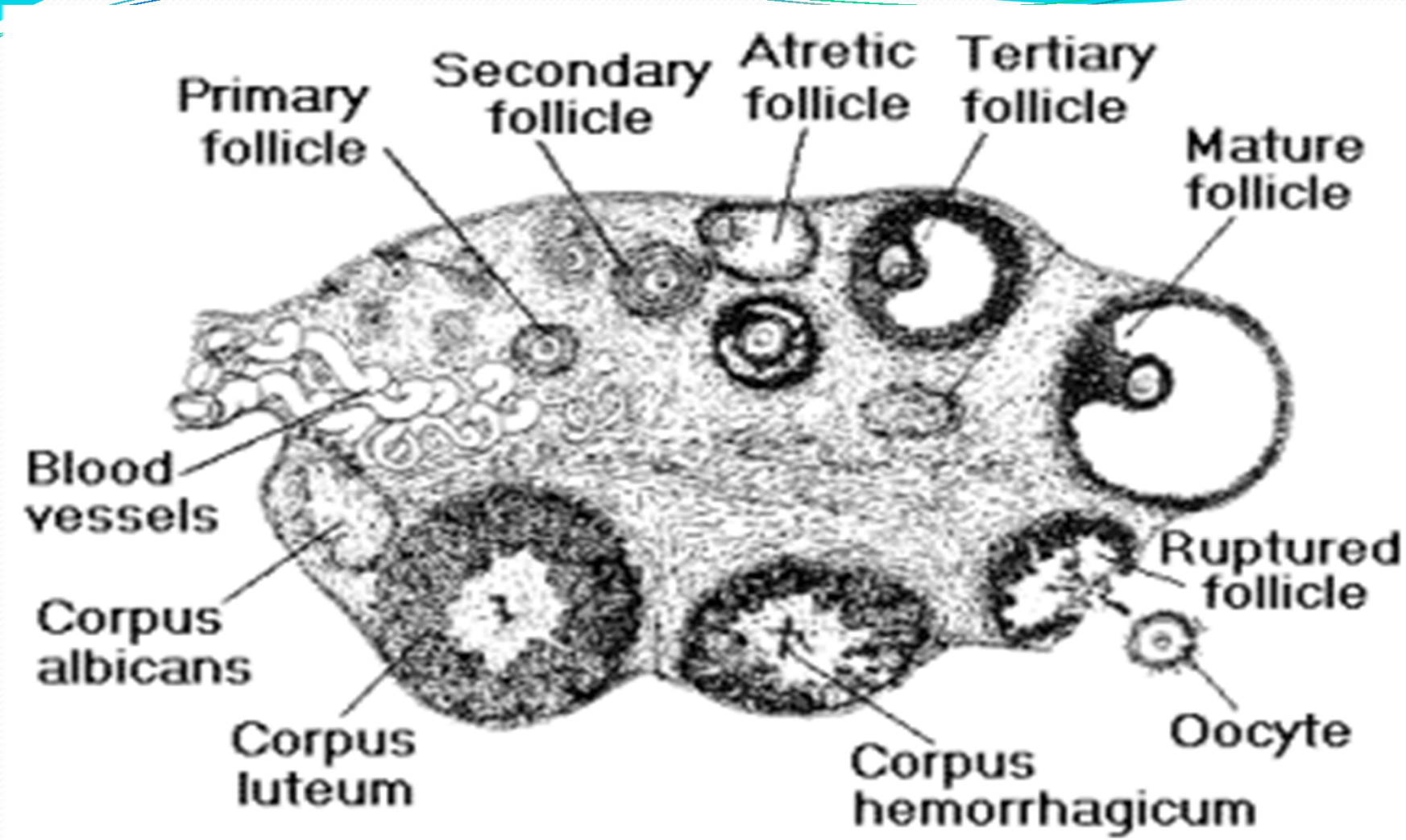


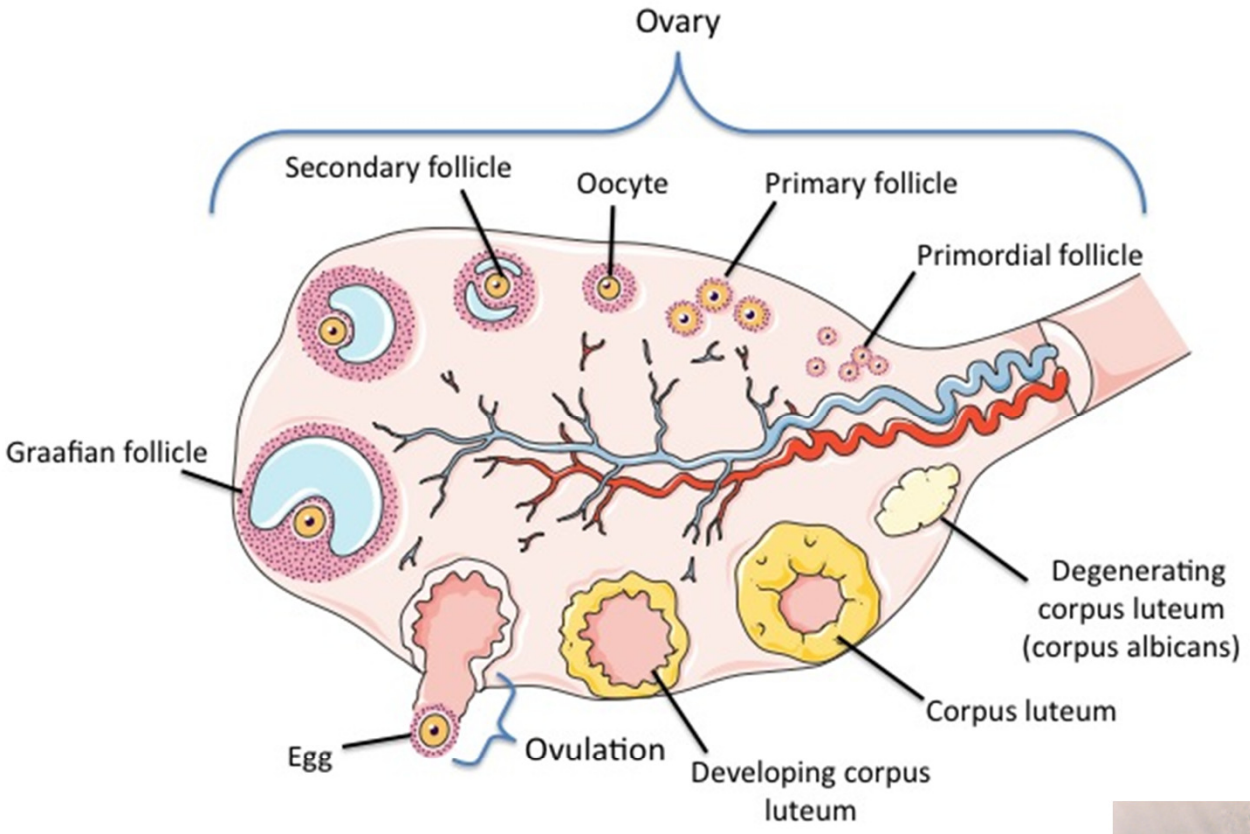




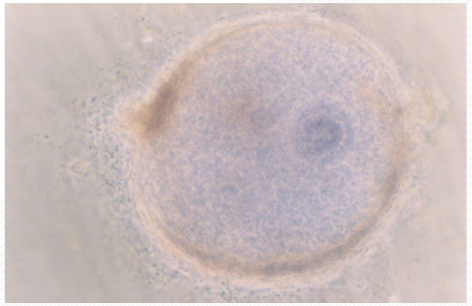


In vitro culture of pre-antral follicles for production of Fertilizable oocytes

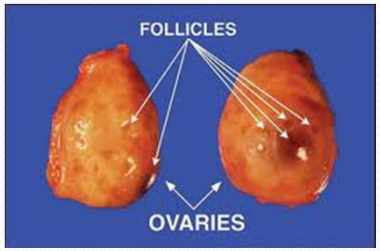

Primary follicle
Secondary follicle
Atretic follicle
Tertiary follicle
Mature follicle
Blood vessels
Ruptured follicle
Corpus albicans
Corpus luteum
Corpus hemorrhagicum
Oocyte



Figures were produced using Servier Medical Art: www.servier.com

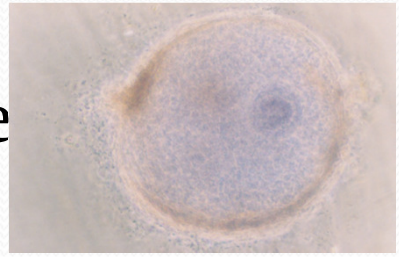


Production of embryos using oocytes from PF



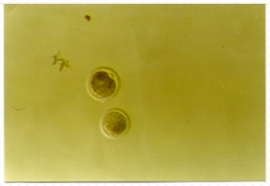
Ovarian cortex

Preantral follicle



In vitro culture of PFs with Somatic cells / GFs/ hormones

Isolation of oocytes from In vitro grown PFs





IVF

Oocytes for Transgenics

Embryos for transfer / cryopreservation

Isolation and culture of large preantral follicles

Ovarian cortex

↓

ovarian cortical slices (1mm)

↓

Incubated with 1 % trypsin for 10 minutes

↓

PF separated by microdissection method

↓

Viability by 0.05 % trypan blue staining for 2 min

↓

Diameter measured in stereozoom microscope

↓

Cultured in 100 µl culture medium

Cultured under mineral oil in co_2 incubator

Growth rate on day 0 and on every 10 days

Vitality and morphology on days 0, 30, 60,80 and 100

- Fresh cells / medium added every alternate days

Culture medium:

MEM, steer serum (10%), FSH (0.05 IU/ml), ITS (1%),

ME (10µM/ml),sodium pyruvate (0.23 mM), glutamine

(2mM), hypoxanthine(2mM), gentamycin (50µg/ml)

Isolation of oviductal epithelial cells:

Oviduct with growing CL ovary

↓

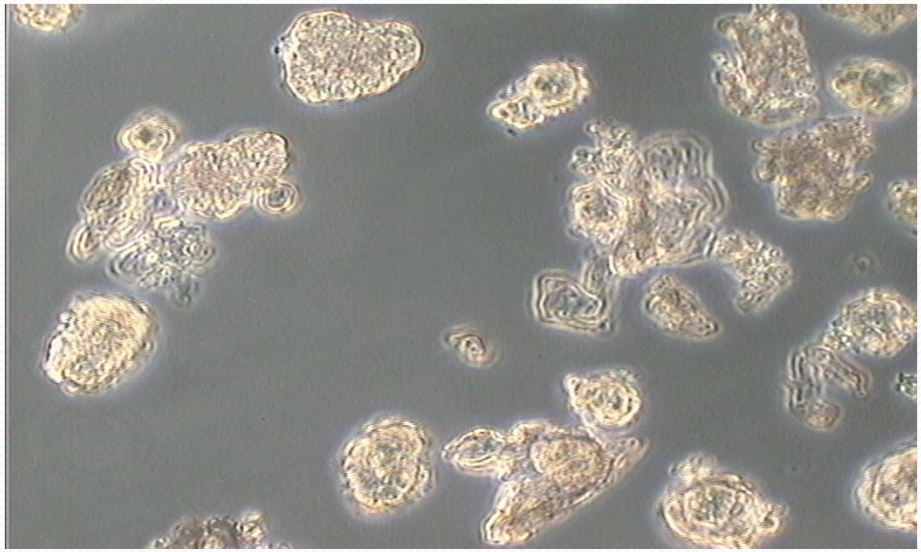
Squeezing

↓

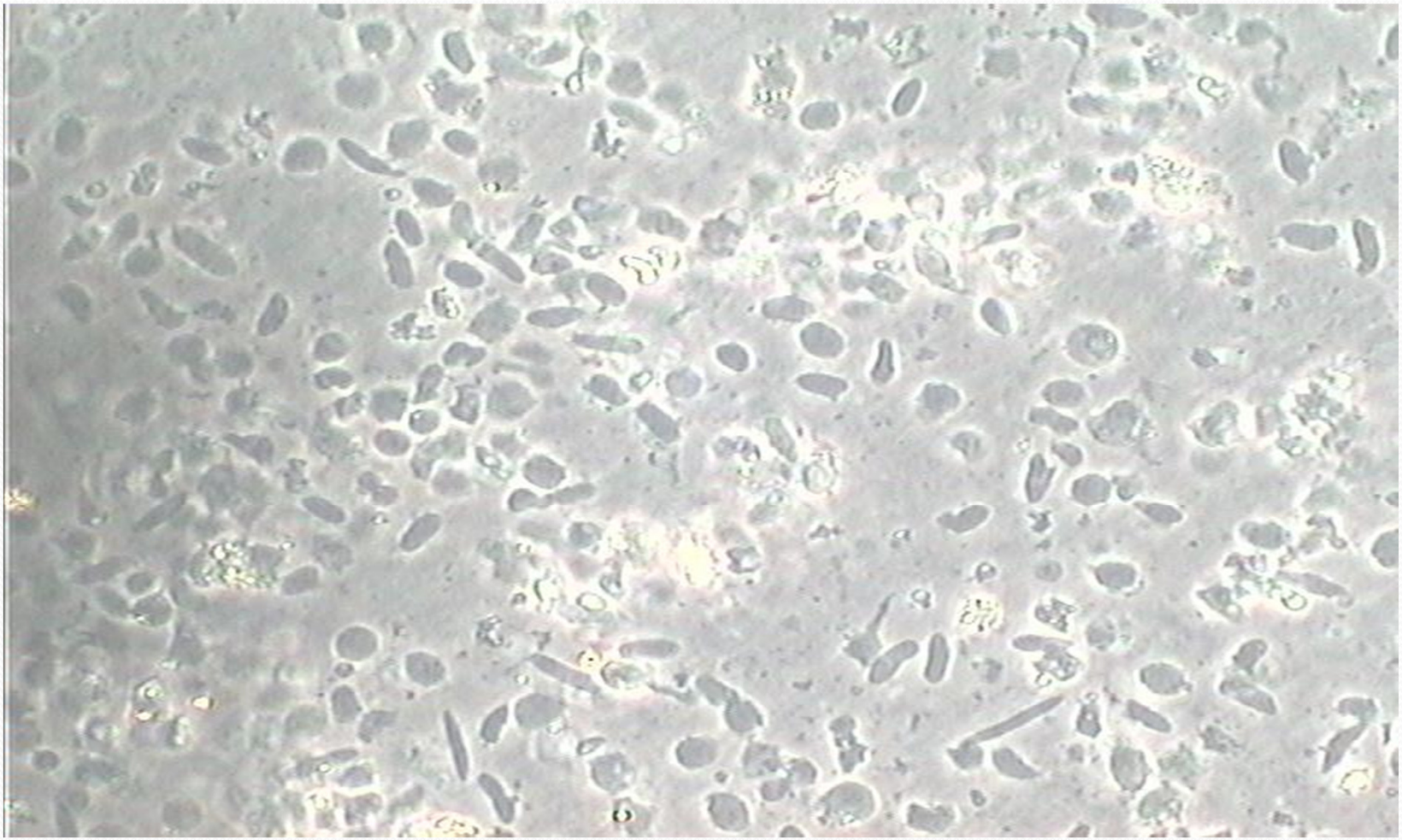
Centrifugation at 5000 rpm for 10 min

↓

OEC

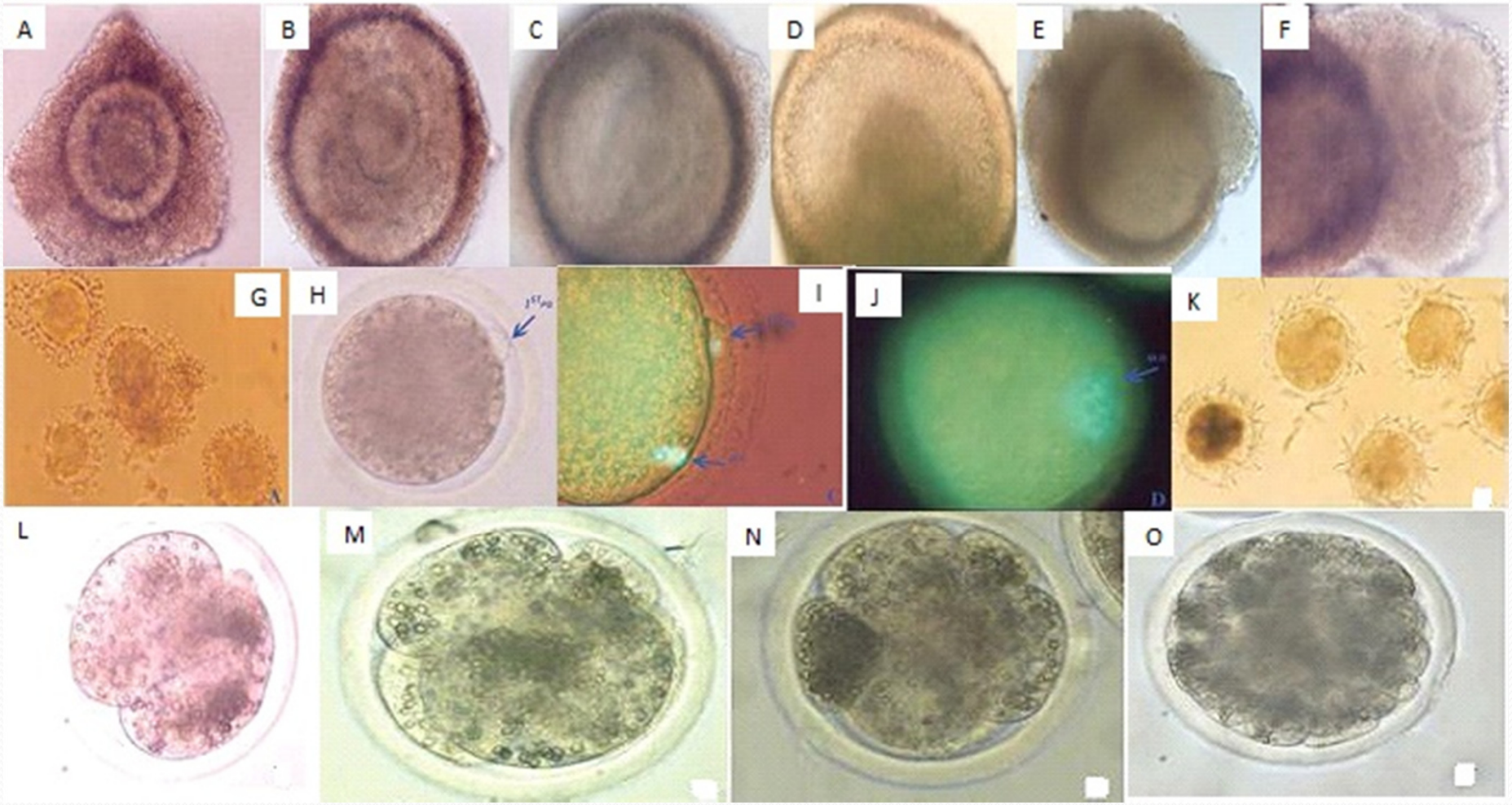






Buffalo ovarian Mesenchymal cells

Isolation And Embryo Production From Preantral Ovarian Follicles



Sexing of Sperm

- **Physical, biochemical, and immunological differences,.**
- **Albumin gradients,**
- **Percoll® gradients,**
- **Sephadex columns, and**
- **Modified swim-up,**

All subsequently resulting in unconfirmed sperm cell separation after DNA analysis and inconsistent gender reports (Flaherty and Matthews, 1996 and Reubinoff and Schenker, 1996).

Spermatozoa differs in sex chromosome size

recognizable distinction between living X- and Y-bearing sperm is their total DNA content, due to the larger size of the X chromosome

- **Cytometry is a technique for making measurements by microscopic methods.**

- **FLOW cytometry -measurements are performed whilst the cells flow past an array of optical detectors.**

- **Flow cytometry measurements that could be made by (fluorescence) microscopy:**

- **Fluorescence from added compound(s)**

  Different dyes specific for a variety of cellular componenets will provide information related to different cellular parameters

How do flow cytometers work?

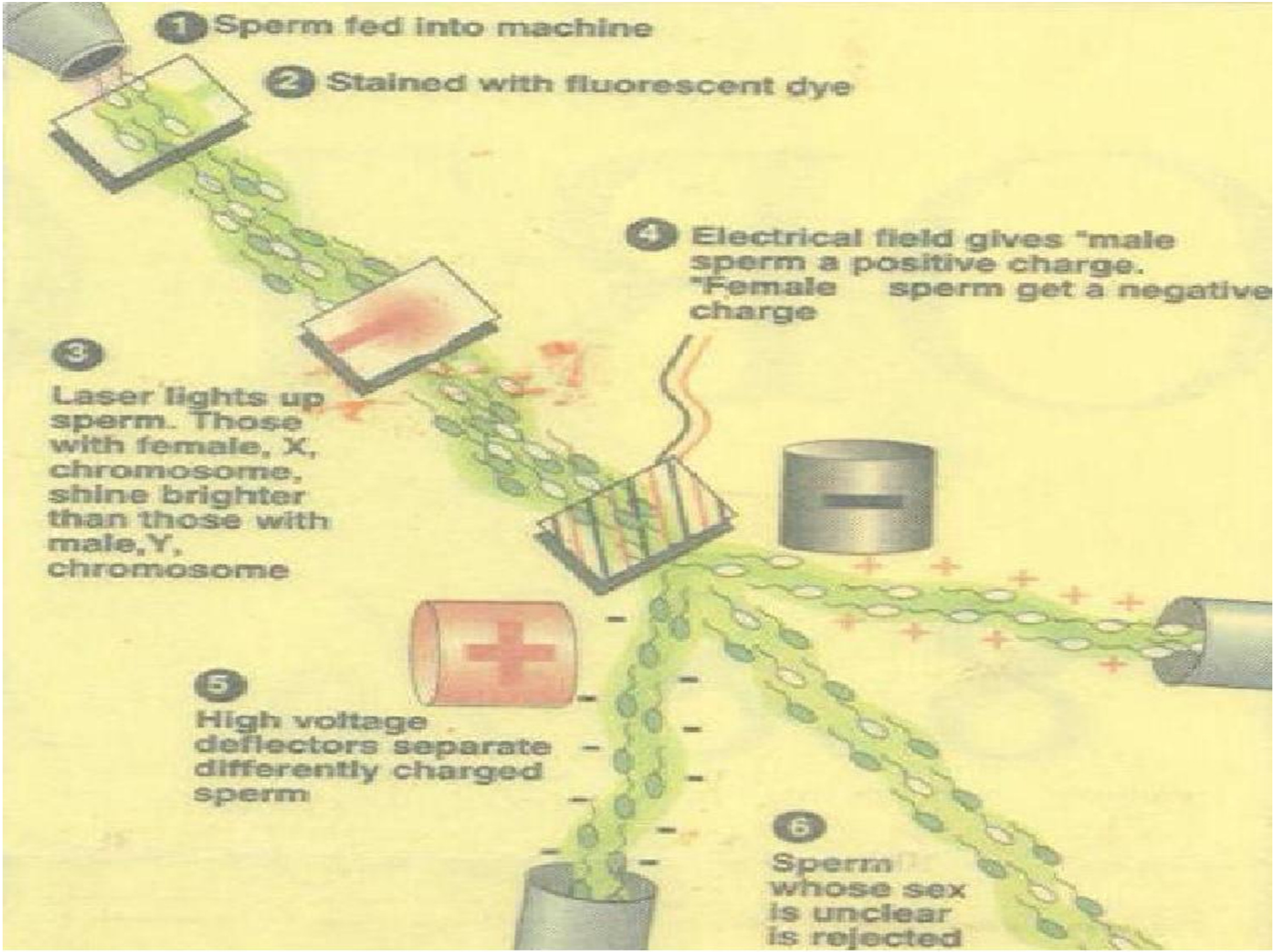
- The fluidics system which is required for sample delivery
- The optical system which makes the measurements
- The electronics used for signal detection, data processing and automation
- The computer interface - i.e the software and hardware used to control the flow cytometer and to collect, display and store the data

Why use flow cytometry?

- Conventional microscopy is labour intensive and slow
- Microscopic analyses are qualitative or at best semi-quantitative
- flow cytometry is a **rapid** technique
- Flow cytometry uses very sensitive electronic detectors called photomultiplier tubes to measure the intensity of scattered light or fluorescence at a given wavelength

Modern flow cytometric cell sorting instrument

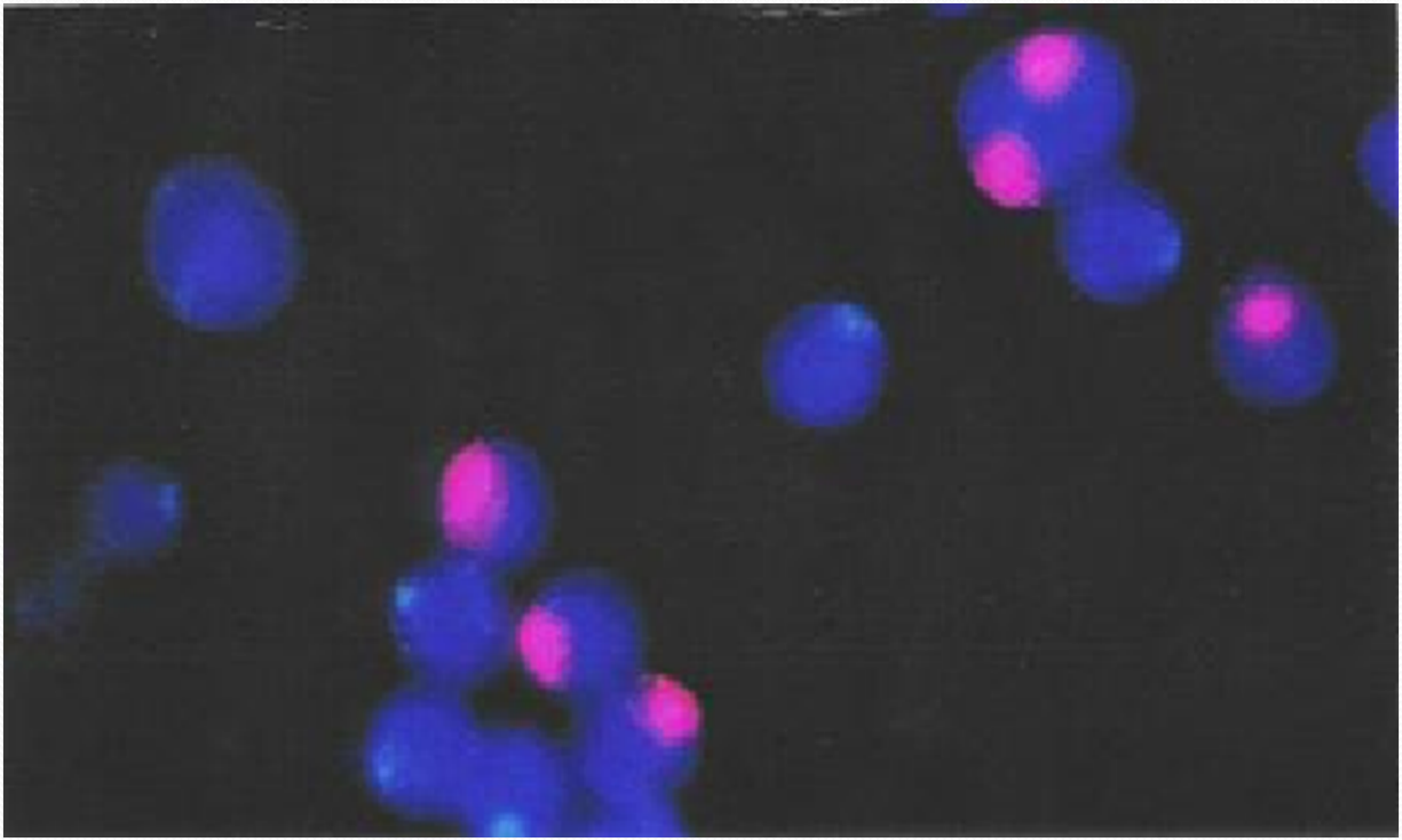


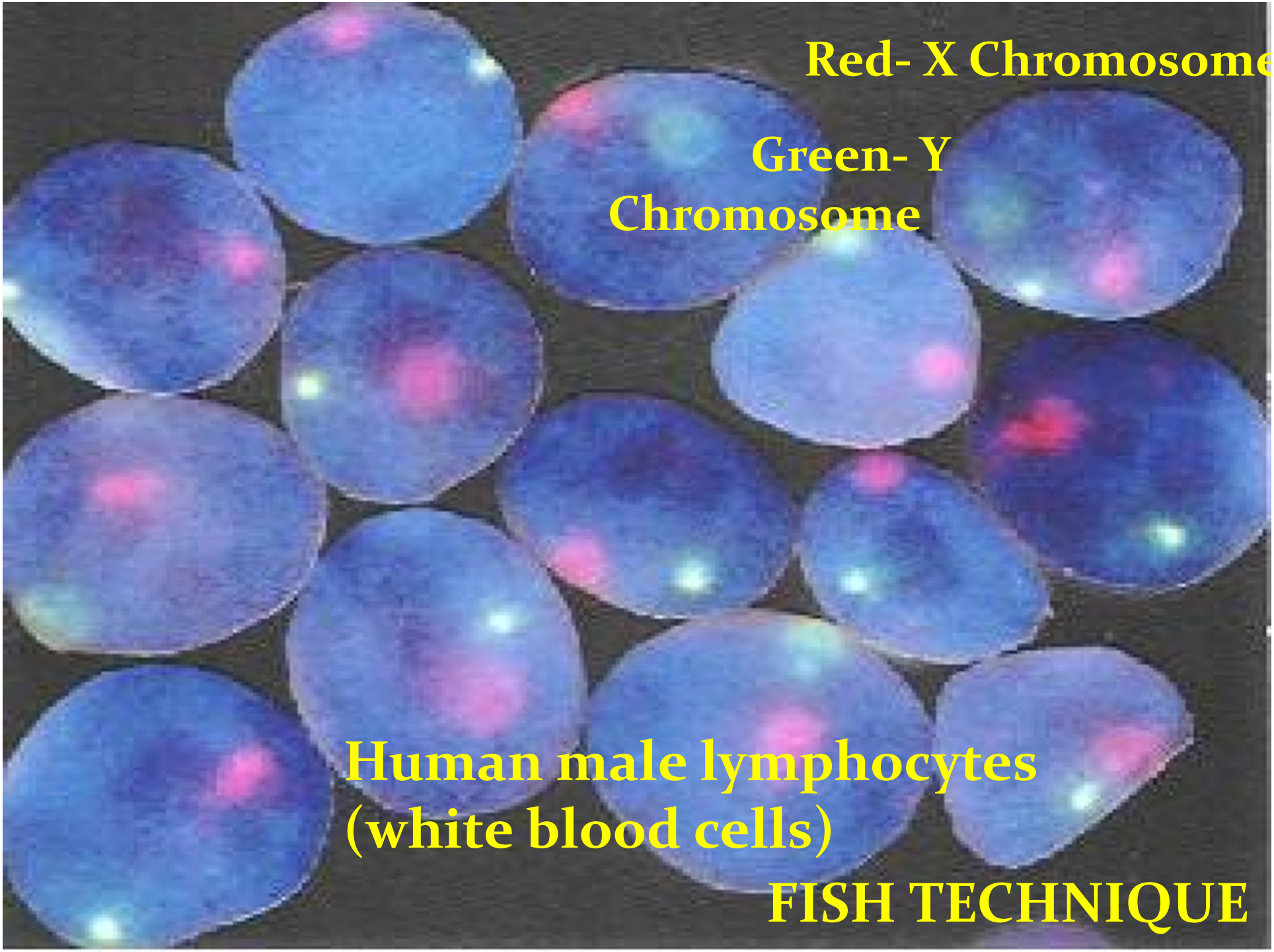

1 Sperm fed into machine
2 Stained with fluorescent dye
3 Laser lights up sperm. Those with female, X, chromosome, shine brighter than those with male, Y, chromosome
4 Electrical field gives "male sperm a positive charge. "Female sperm get a negative charge
5 High voltage deflectors separate differently charged sperm
6 Sperm whose sex is unclear is rejected

SORTING SPERMS- FISH

- With the commercial availability of directly labelled multicoloured fluorescent DNA probes for chromosomes X and Y, a highly reliable molecular biology technique known as Fluorescence in-situ hybridisation (FISH) to analyse and quantify the percentage distribution of the X- and Y- sperm in human semen.
- In this method, the sperm cells are first pre-treated to de-condense the sperm nuclei and make "holes" in the sperm head so that specific DNA probes can gain access to the genetic material of interest (i.e. the X and Y chromsomes).

SORTING` X / Y CHROMOSOME BEARING SPERMS




Red- X Chromosome
Green- Y Chromosome
Human male lymphocytes (white blood cells)
FISH TECHNIQUE

Sexing of Embryos

- cytogenetic analysis
- assays for X-linked enzyme activity
- analysis of differential development rates
- detection of male-specific antigens

by Y-specific DNA probes.

PCR amplification of sex-specific DNA sequences to determine mammalian embryonic sex is most commonly used technique

(Tominaga, 2004).

A fast and reliable method for bovine sexing has been developed through amplification of the bovine high motility group (HMG) box of the sex-determining region of the Y chromosome gene(SRY)

(Lu *et al*.,2006).

An another stable, simple and fast method

two temperature gradient PCR

takes 57 min. to identify sex of the embryo.

-Wang et al., (2006)

Murine and bovine embryos at the late morula stage were cultured in medium containing high-titer rat H-Y antisera.
After 12h of incubation, embryos blocked at the late morulae stage were classified as males and those at the blastocyst stage were classified as females.
Sexing of murine embryos by PCR and cytogenetics revealed that 83% of the embryos classified as males and 82% of those classified as females had their sex correctly predicted ($P < 0.05$).

(Ramalho *et al*.,2004)

Rapid sexing of bovine preimplantation embryos using loop-mediated isothermal amplification.

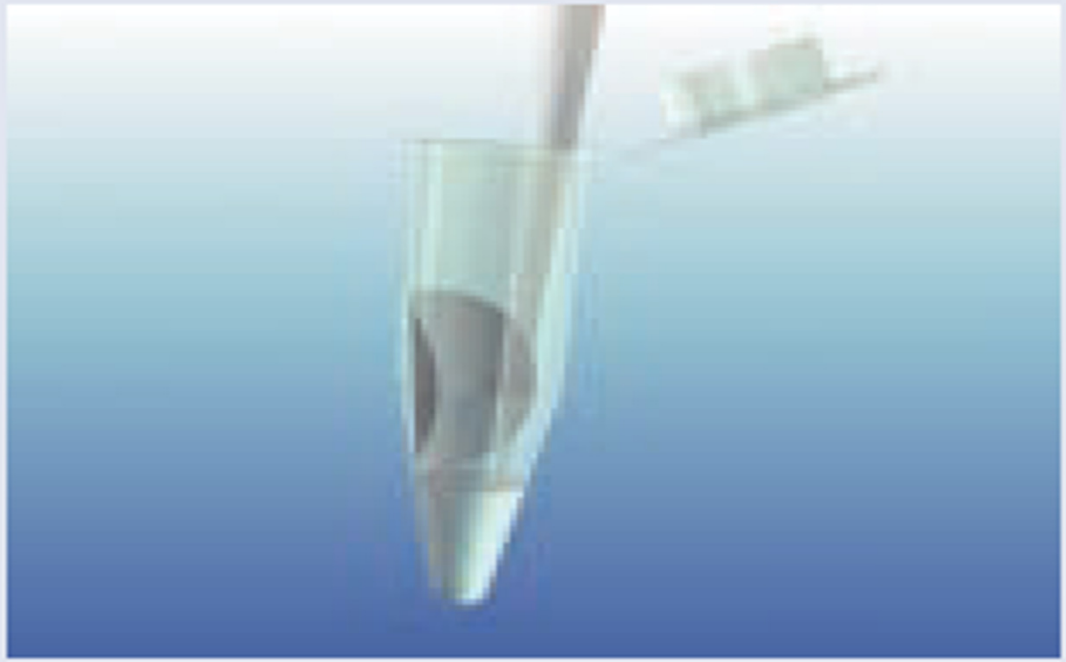
Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method that amplifies a target sequence specifically under isothermal conditions. The product of LAMP is detected by the turbidity of the reaction mixture without electrophoresis

- Hirayama *et al.*,2004

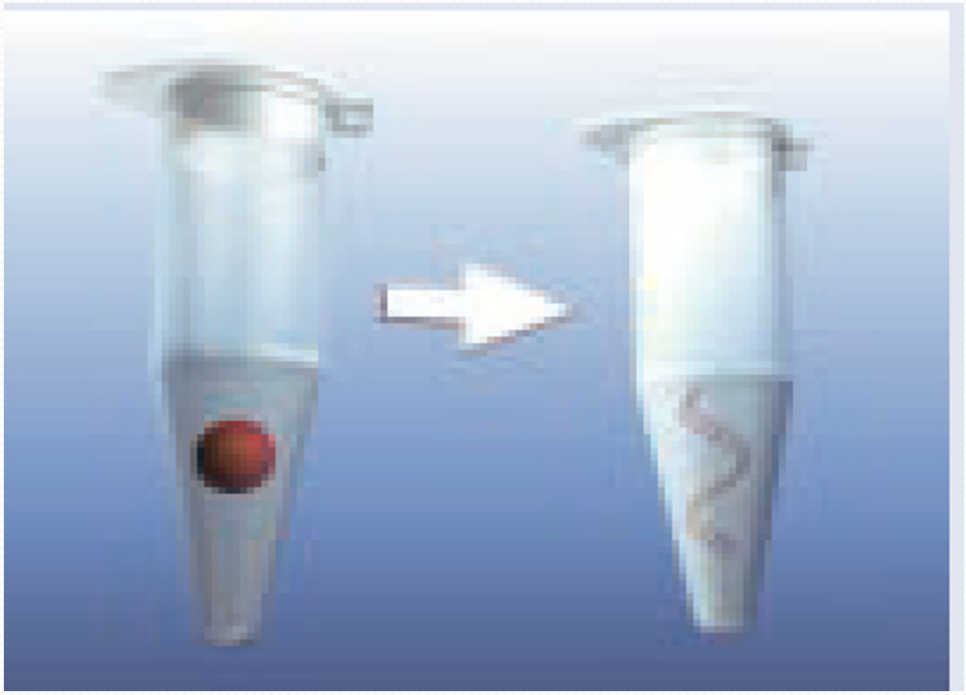

Rapid non-electrophoretic
sex determination
of bovine embryos

1. Transfer biopsy into tube

Each biopsy is transferred in 2µl biopsy medium into a reaction tube prefilled with 10µl lysis mixture. Note the piece of wax on the left inside wall.



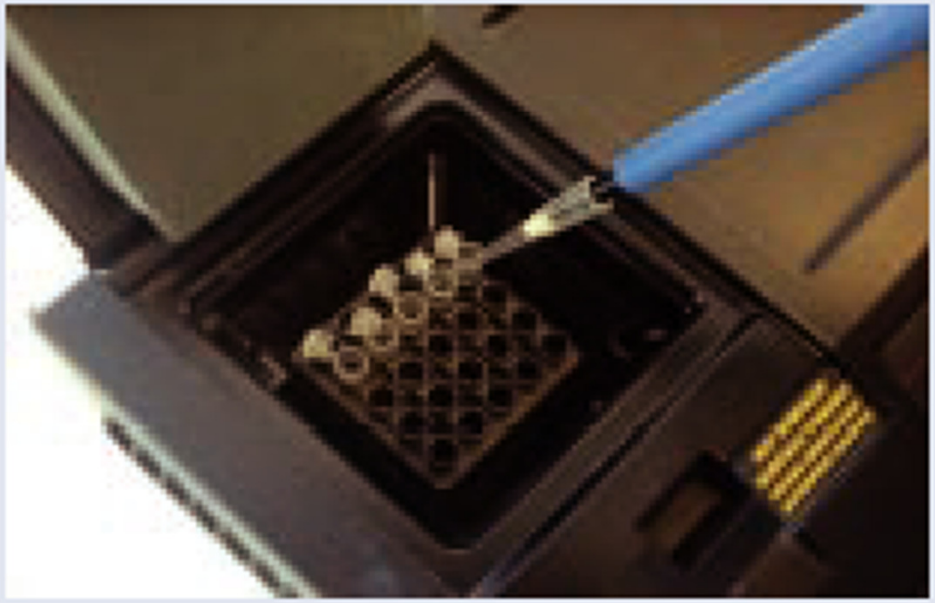
DNA release



2. Lyse cells in PCR cycler

The tubes are placed in the PCR cycler (5 min at 55°C, 5 min at 95°C). DNA will be released and the wax will melt and form a layer preventing evaporation.

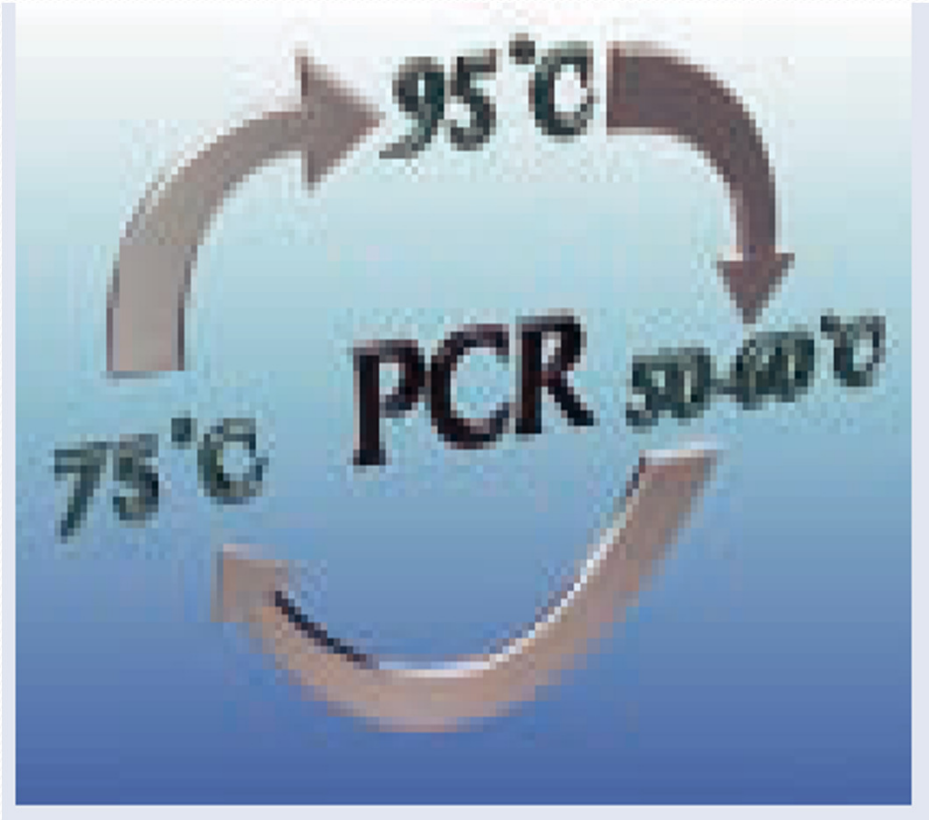




3. Add B-mix and run PCR

The PCR program is started and 15 µl of amplification mixture is added into each tube. The program takes about 70 minutes.

PCR cycling





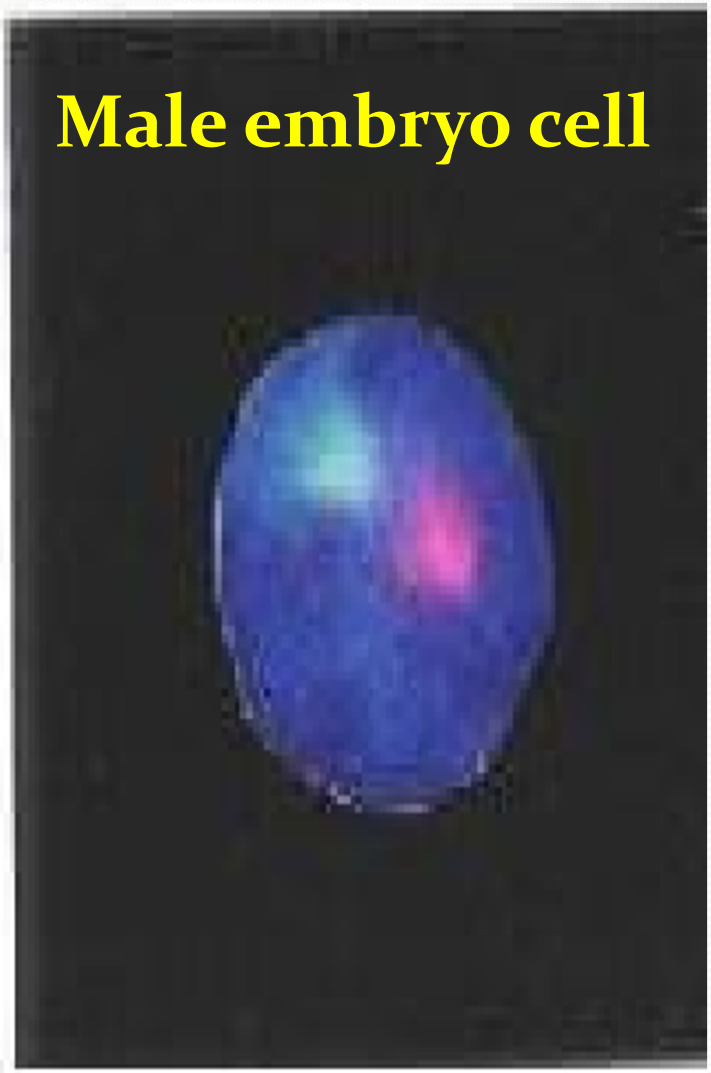
4. Inspect tubes to determine sex

The tubes are taken out of the PCR cycler and inspected in UV light (preferably 302-312 nm, but 254 nm can also be used). Tubes fluorescing pink contain Y-chromosomal DNA. Female samples do not show fluorescence.
(from left to right: F, F, M, blank, M, M, M)

Female embryo cell



Male embryo cell



CLONING/SCN

Preparation of recipient cell

***In vitro* maturation of oocyte**

Enucleation of oocyte

Artificial activation of oocyte cells

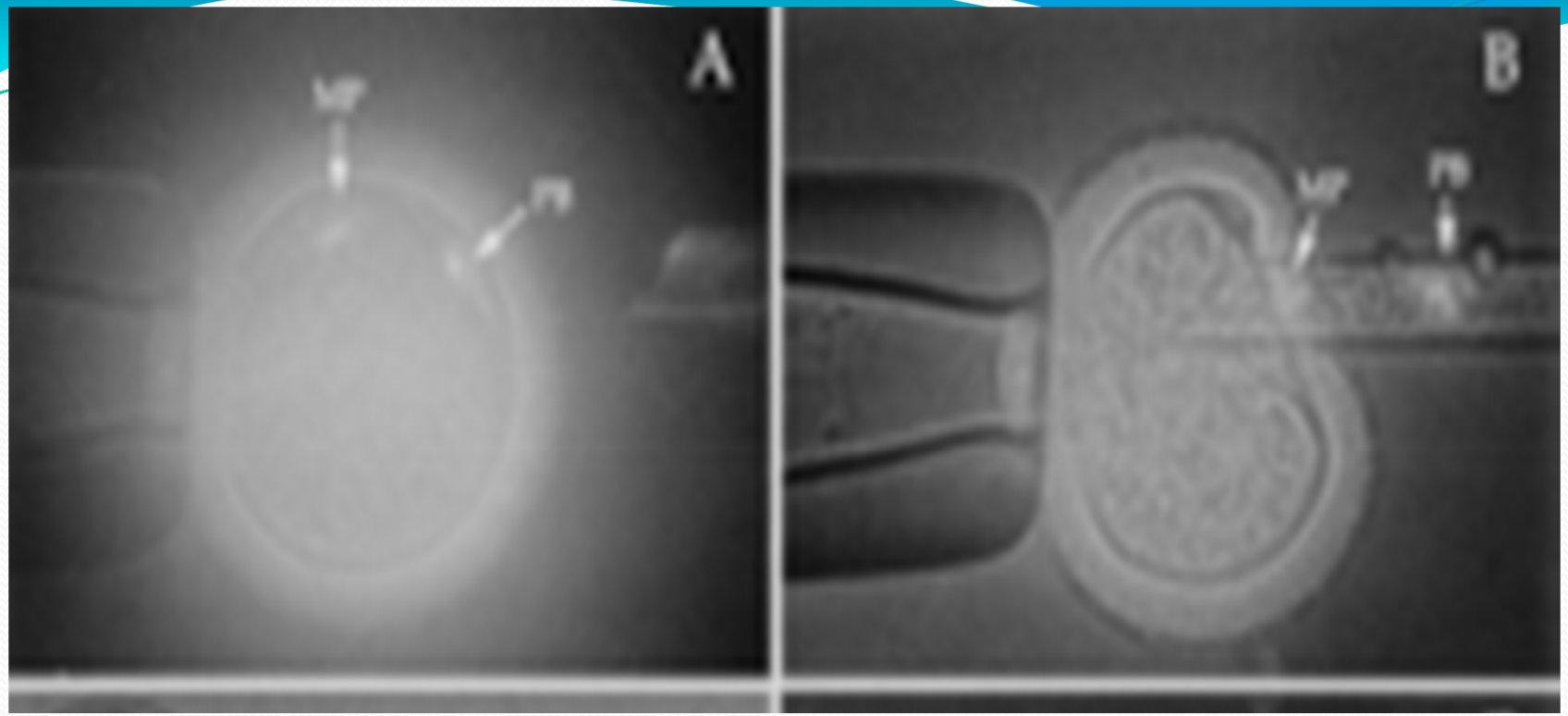
Preparation of donor cell

Serum starvation of donor

Transfer of donor nucleus /cell in to perivitelline space of recipient enucleated oocyte

Fusion of karyo plast with the cytoplast

Culture of embryos up to blastocyst stage in a suitable culture medium



Enucleation of oocyte

A. introduction of microneedle in to perivitelline space

B.Aspiration of metaphase plate (MP) and polar body (PB)



Thank You

