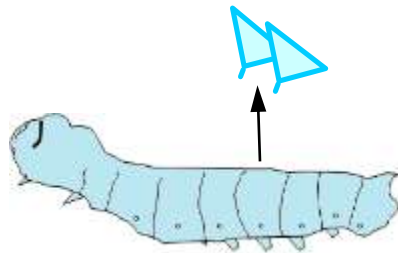


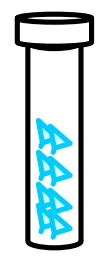
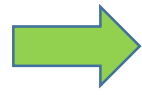
Manual for the cryopreservation of the silkworm germ cells

Yuuji Mochida, Yoko Takemura, Hiroshi Shinbo, Yutaka Banno

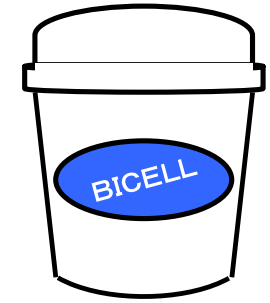
(Published in Sanshi-Konchu Biotec, Vol 83.)



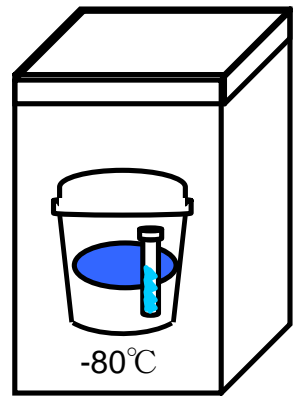
Extirpate ovaries from larvae



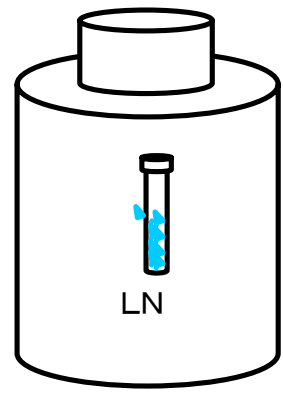
Collect ovaries in Grace insect medium containing cryoprotectant



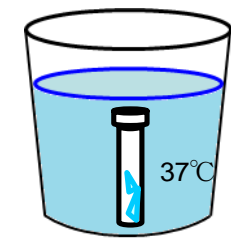
Store ovaries in BICELL (container)



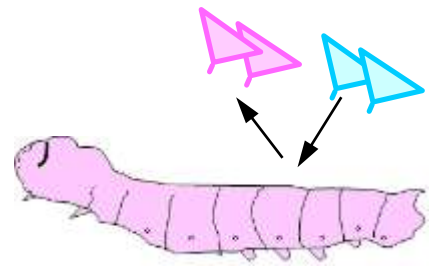
Keep ovaries in freezer



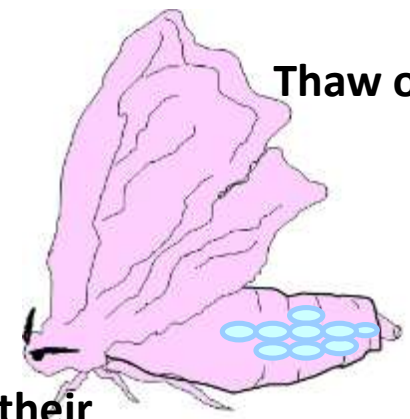
Store ovaries in liquid nitrogen



Thaw ovaries in water bath

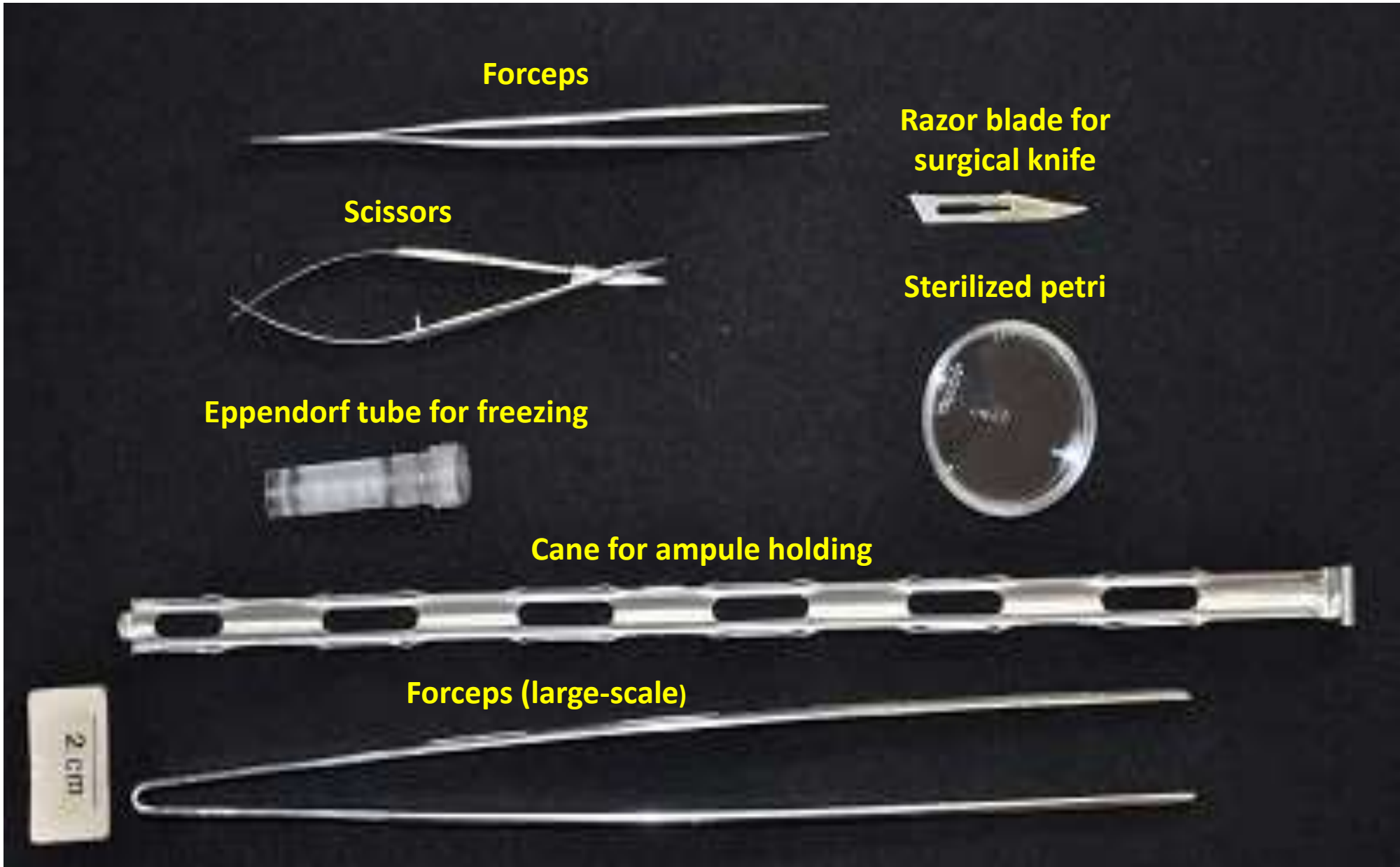


Transplant ovaries into the castrated recipient larva



Rare larvae to complete their life cycle

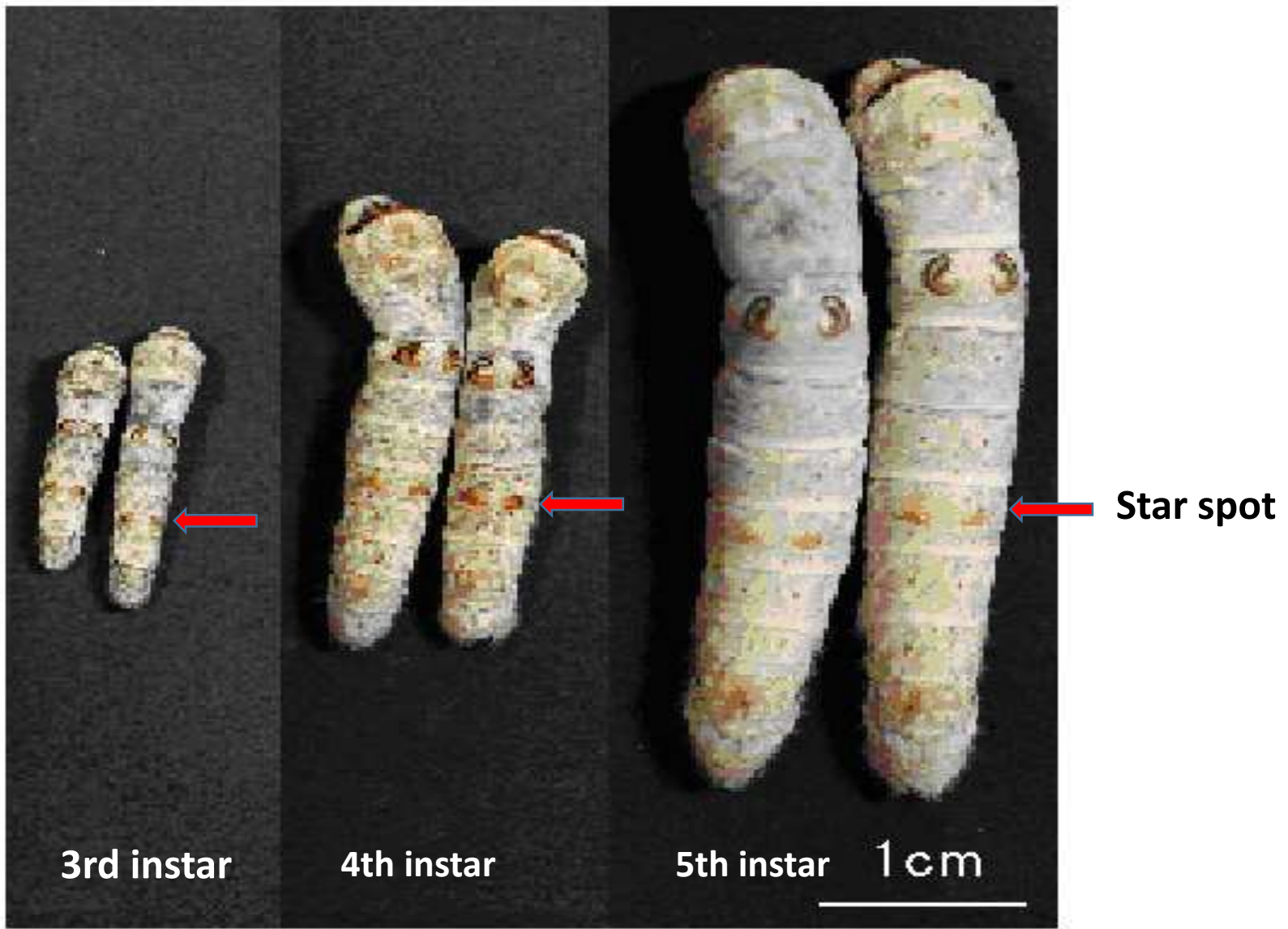
Procedure for cryopreserving the larval ovary



Lab wares used



Manipulation of collection and transplantation of ovaries should be done in clean benches



**Ovaries are located immediately under the “star spots”
on the 8th larval segment**



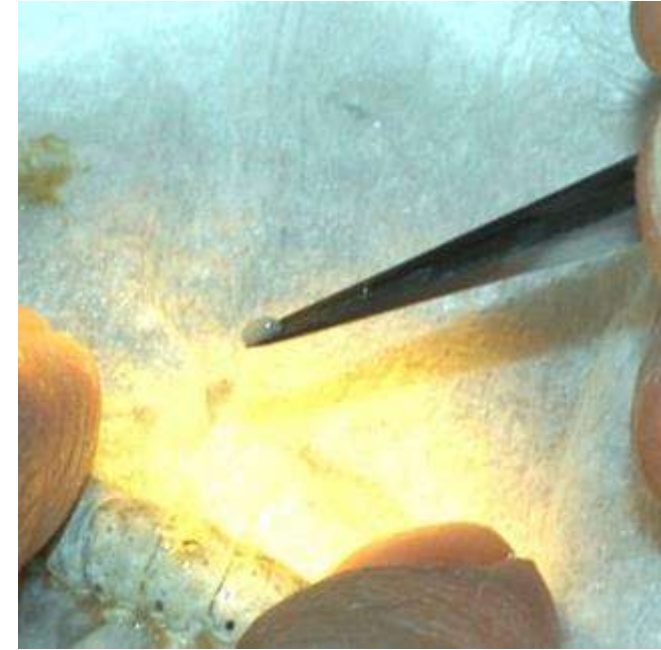
Sterilize body surface in the part of “star spots” with alcohol



**Make small incisions on a pair of
“star spots” with a razor blade**



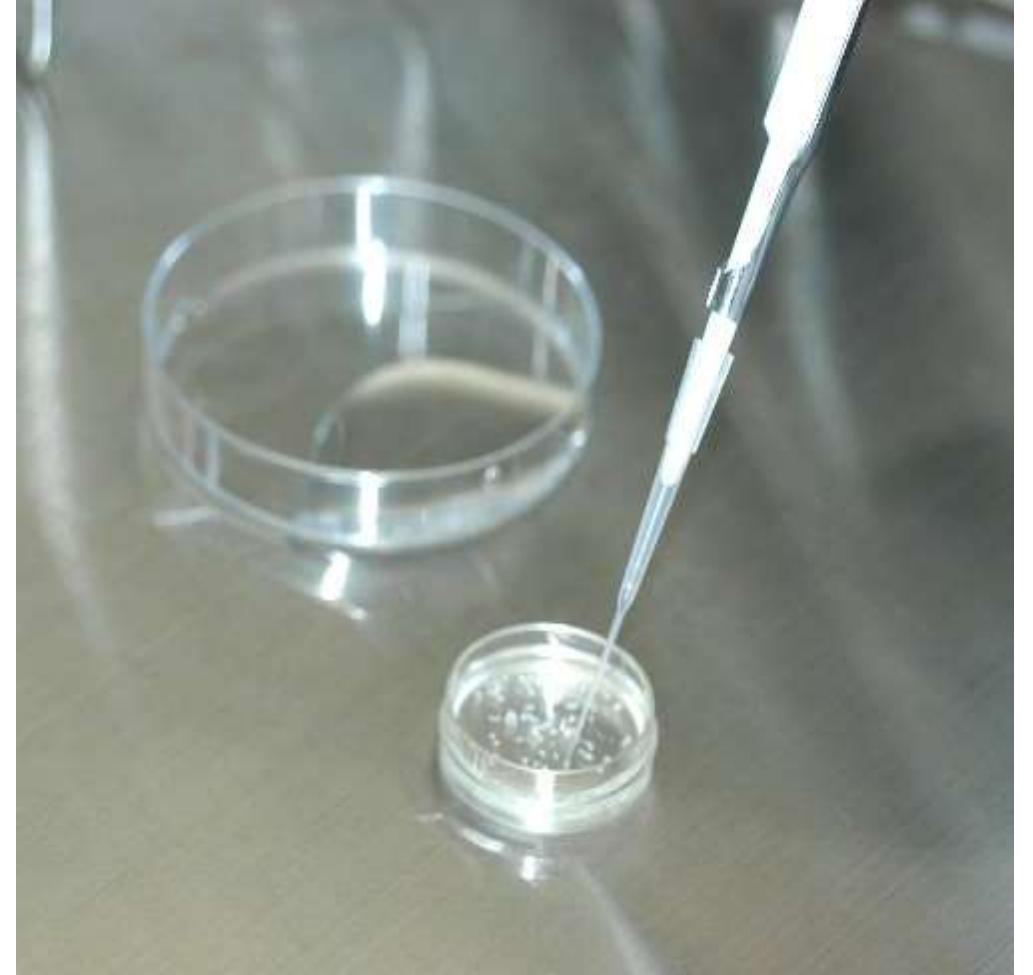
Extirpate ovaries on each side with forceps



Extirpate the larval ovaries



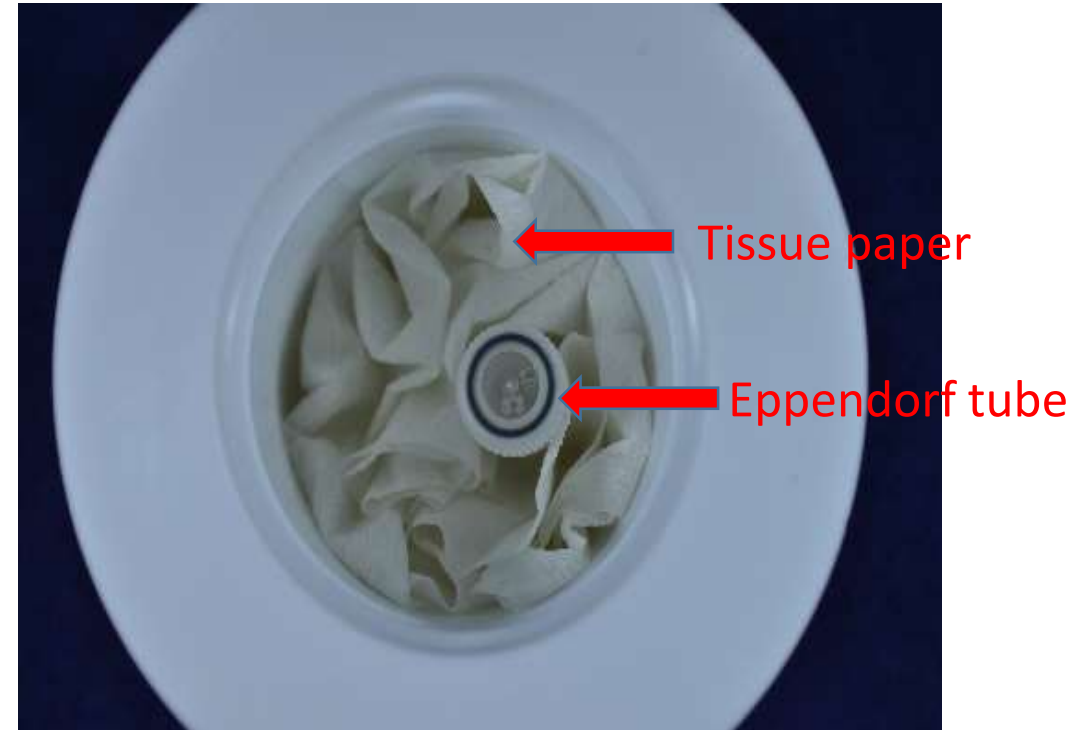
Collect the ovaries in Grace insect medium



Suspend the collected ovaries in Grace insect medium containing 1.5M DMSO as cryoprotectant



**Transfer the ovaries into Eppendorf tube for freezing
by using forceps (A) or pipette (B)**

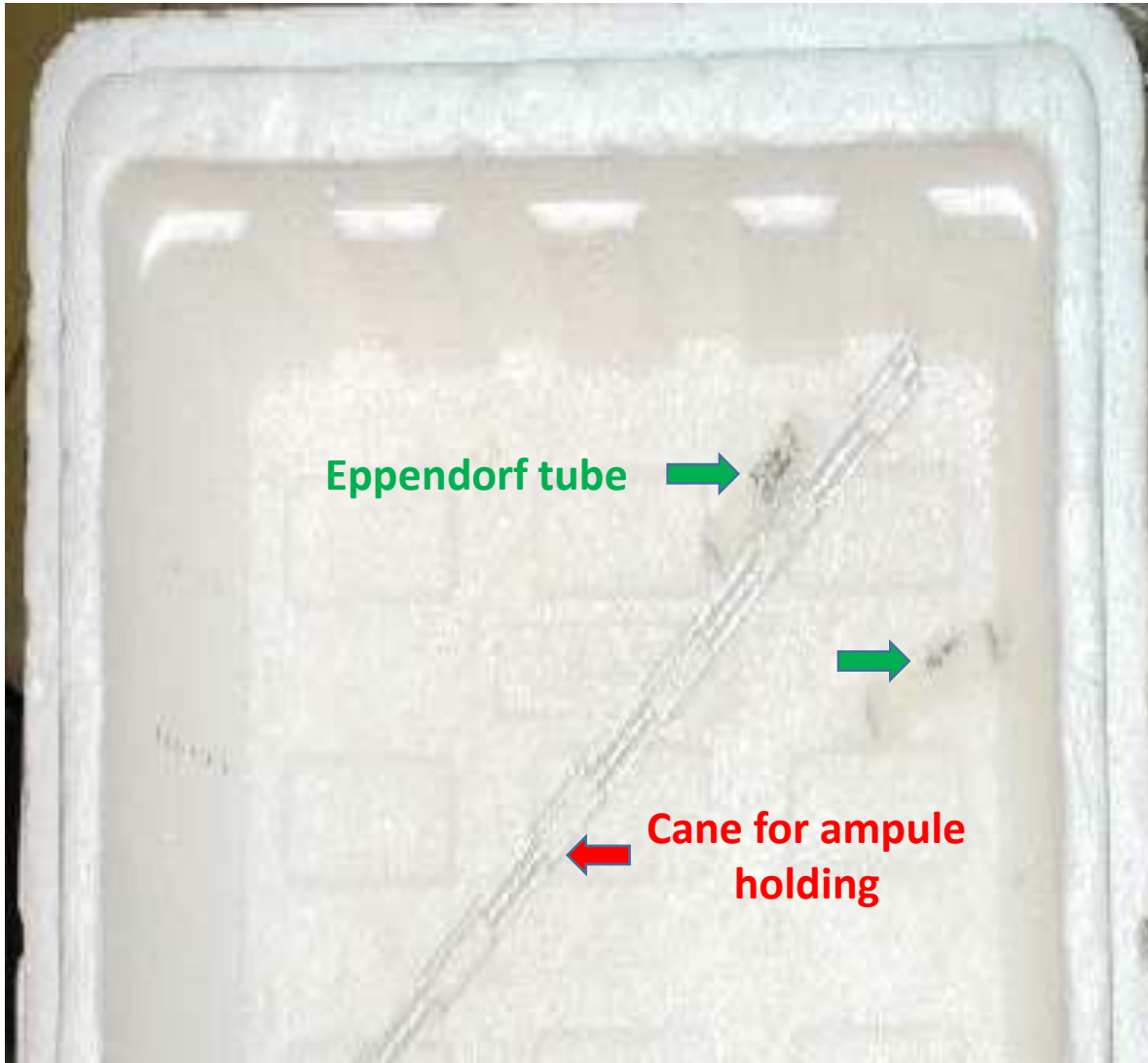


Fix the Eppendorf tube in BICELL (Bio freezing vessel)

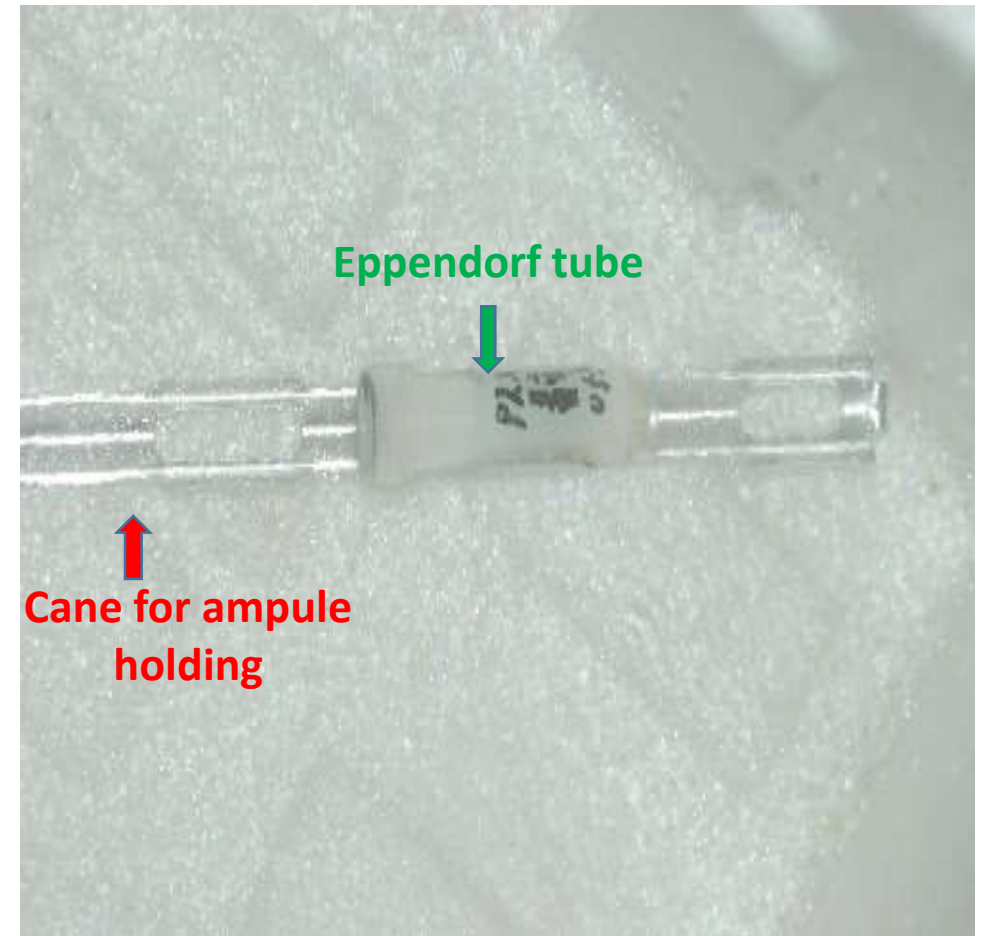


Freezer inside

Keep the BICELL (Bio freezing vessel) in -80°C freezer for ca. 3-20hrs



Fill box of Styrofoam with liquid nitrogen, and place cane for ampule holding in the liquid nitrogen.



Then, attach the Eppendorf tube to the cane for ampule holding in liquid nitrogen by using forceps (large-scale)

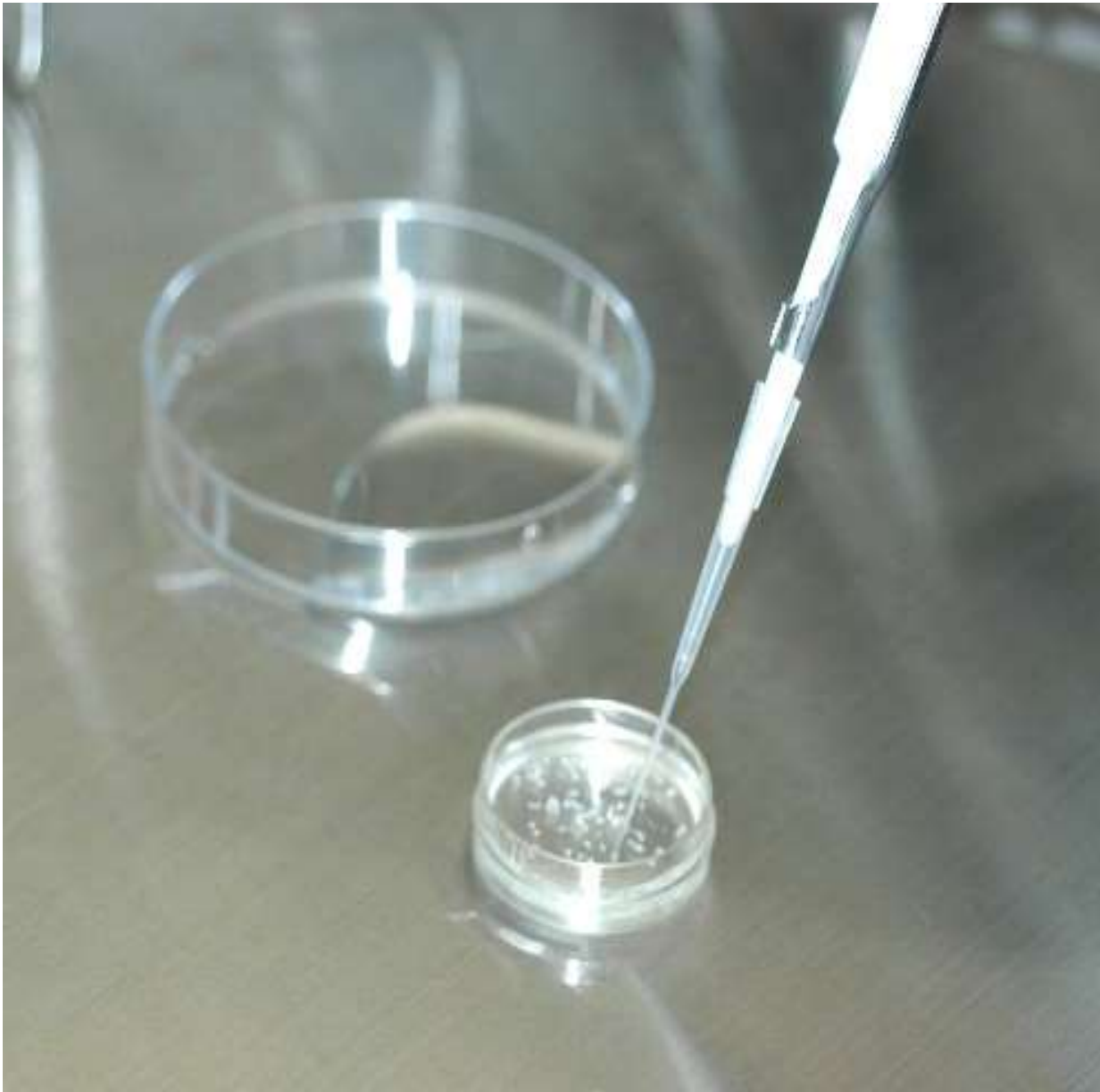


**Mark the tip of the cane
to distinguish samples**

Store the cane in liquid nitrogen preserving container

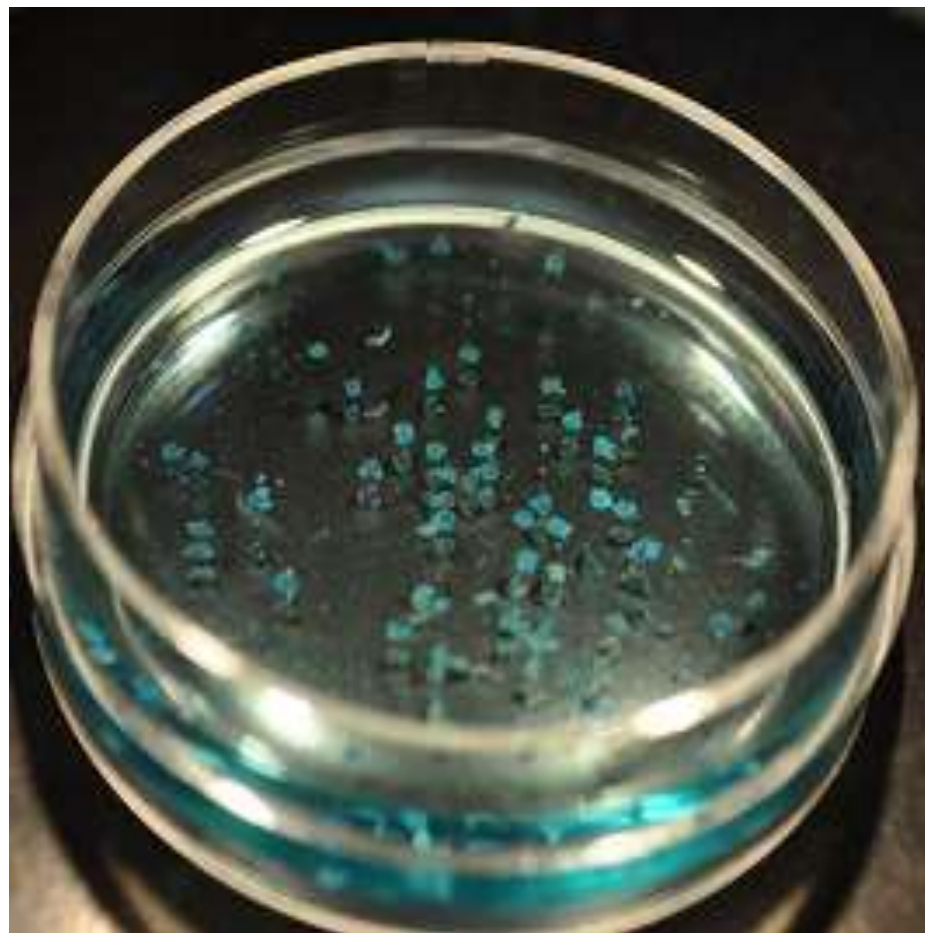


Sift the cane into a 37°C water bath to thaw the ovaries

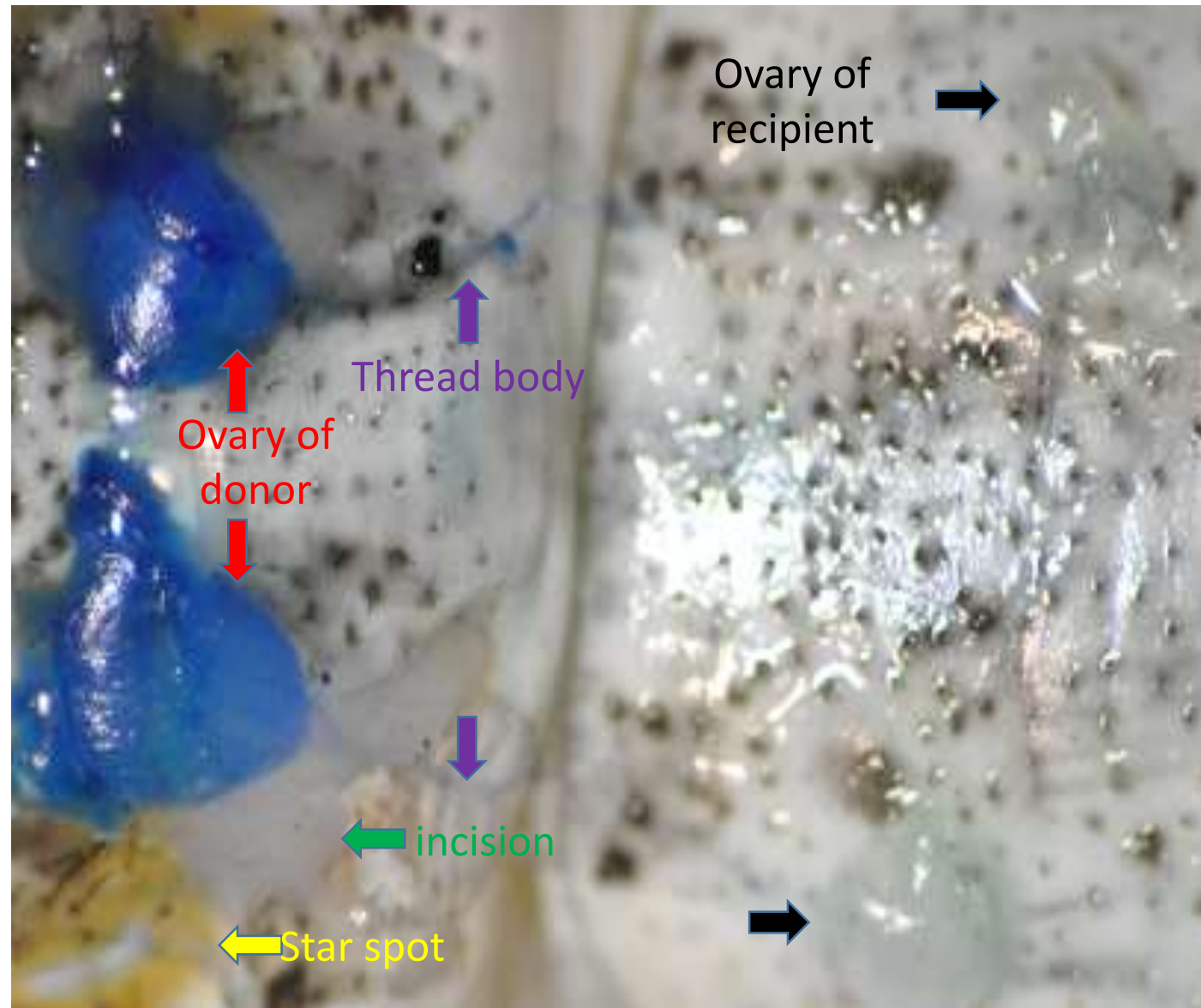


Discard Grace insect medium containing 1.5M-DMSO into petri (upper). Then, pour Grace insect medium into the petri containing ovaries.

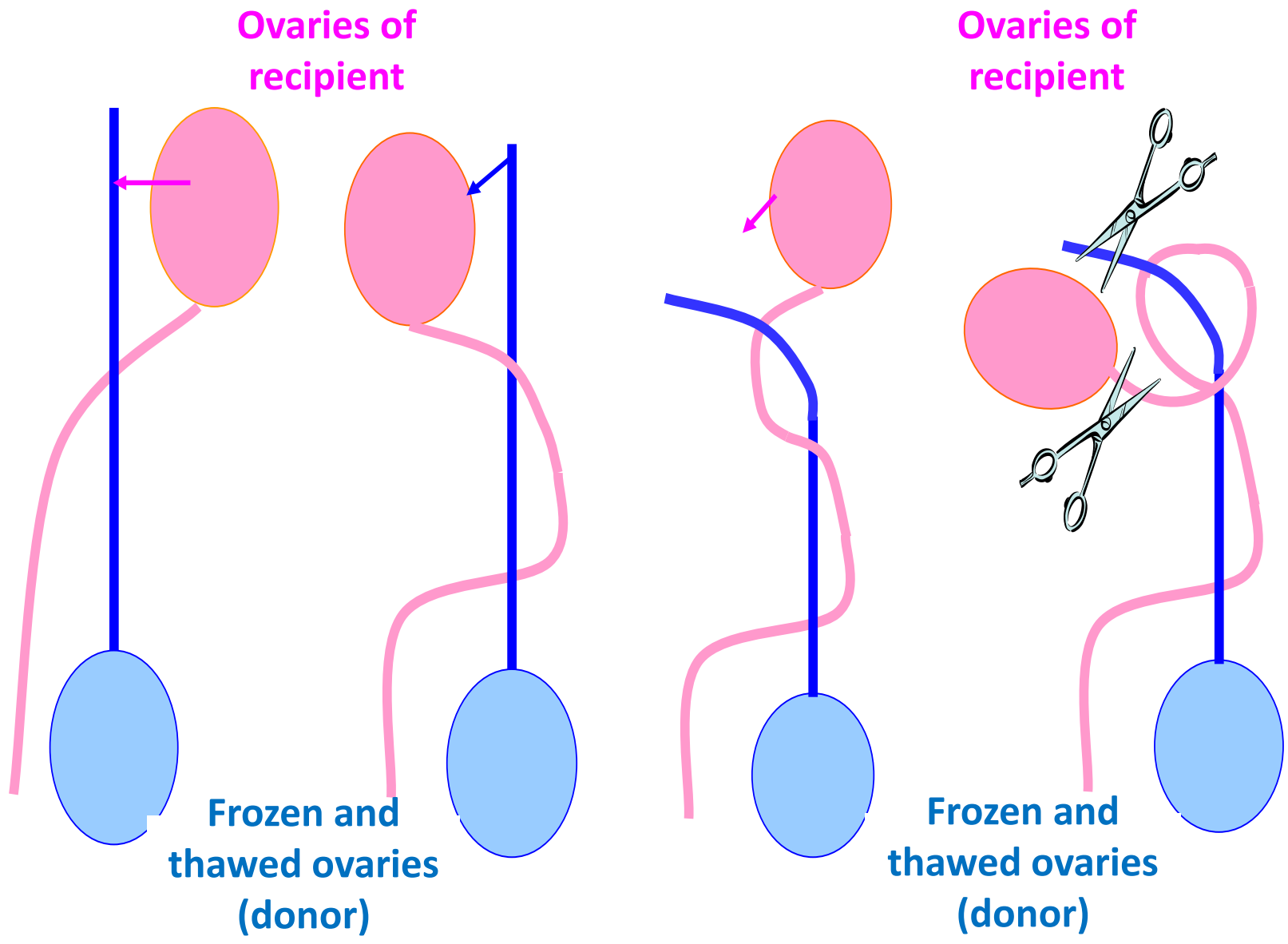
Replace the medium to remove DMSO (cryoprotectant)



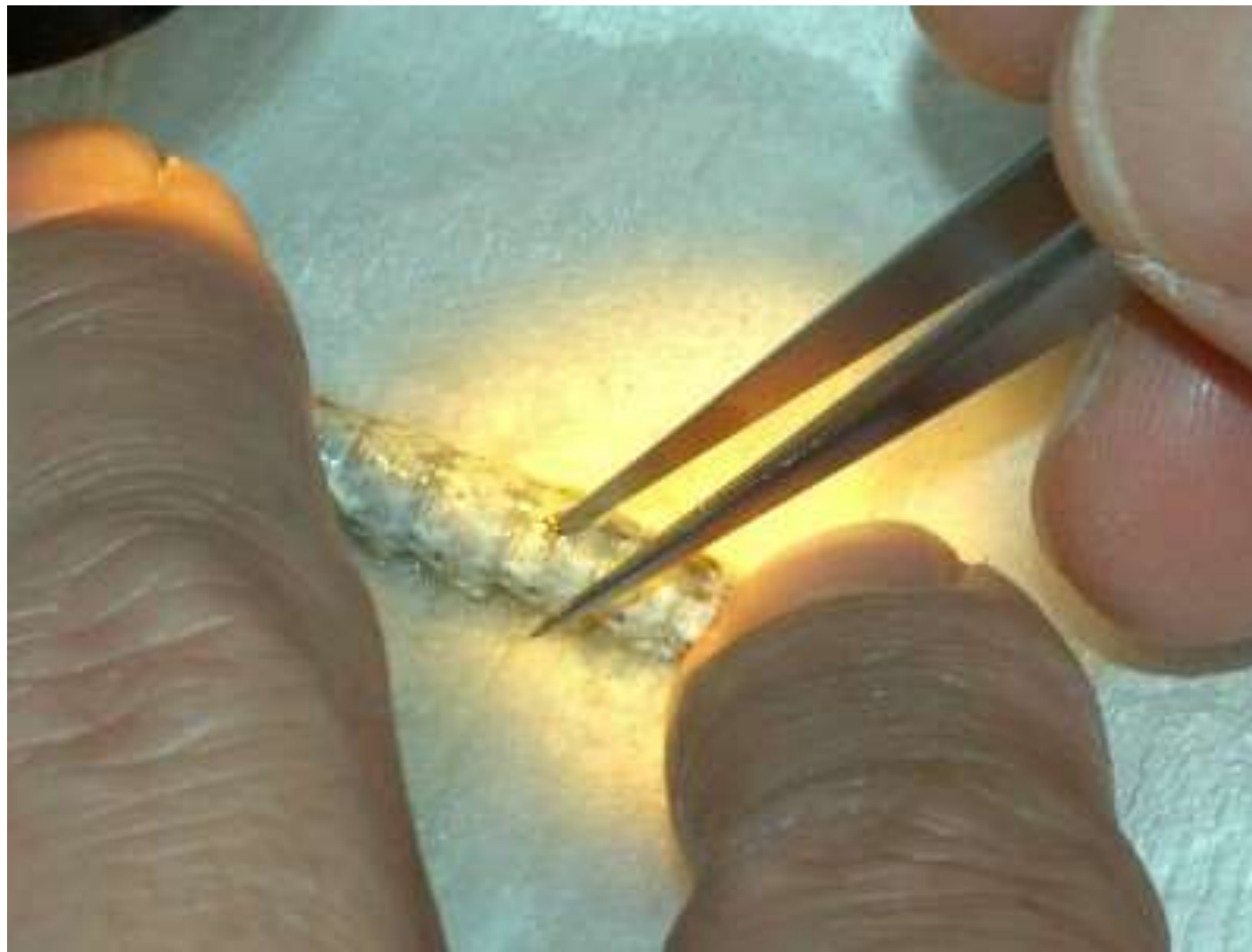
Stain the frozen and thawed ovaries with Nile Blue Sulphate



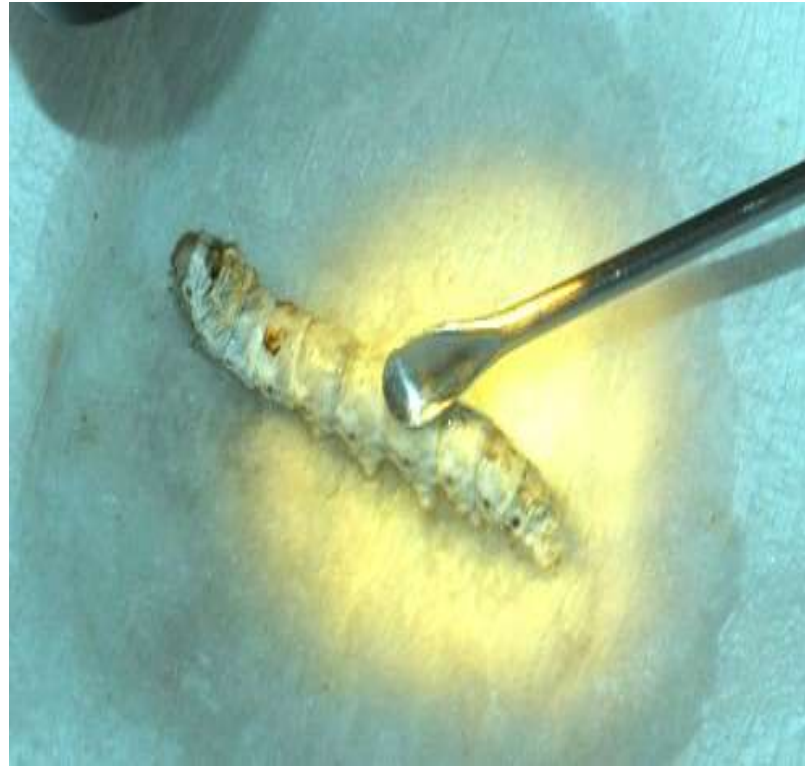
Ovaries of donor (blue) and recipient (milky white translucent)



Connect thread bodies of donor and those of recipient by coiling



Restore the donor ovary with the two thread bodies coiled around one another into the recipient via the original incision



Cover the incisions with paraffin



**2 days after the
transplantation**

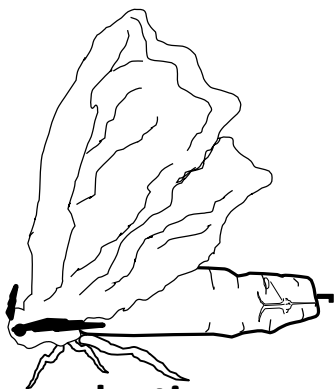


**newly moulted 5th instar
larvae**

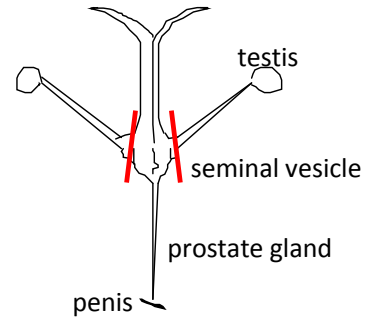


pupae

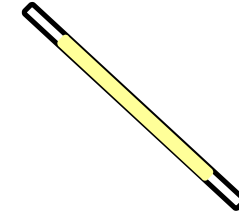
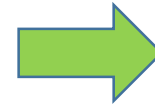
Rear the larvae to complete their life cycle



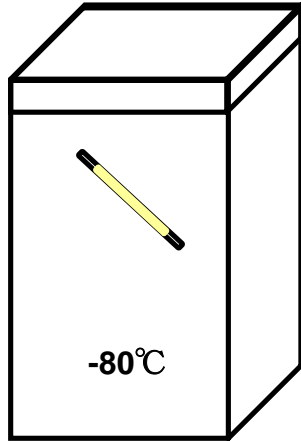
Extract reproductive organs from male moth



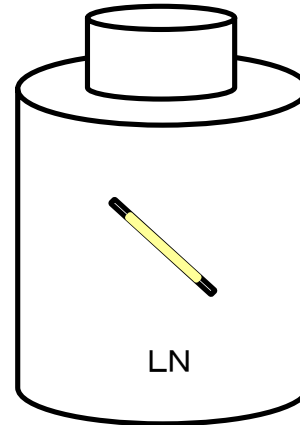
Collect semen from seminal vesicle



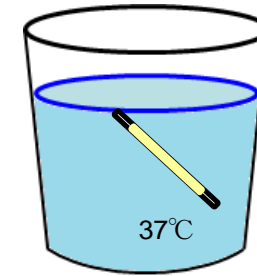
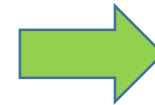
Store semen with cryoprotectant in plastic artificial insemination straw



Keep semen in freezer



Store semen in liquid nitrogen

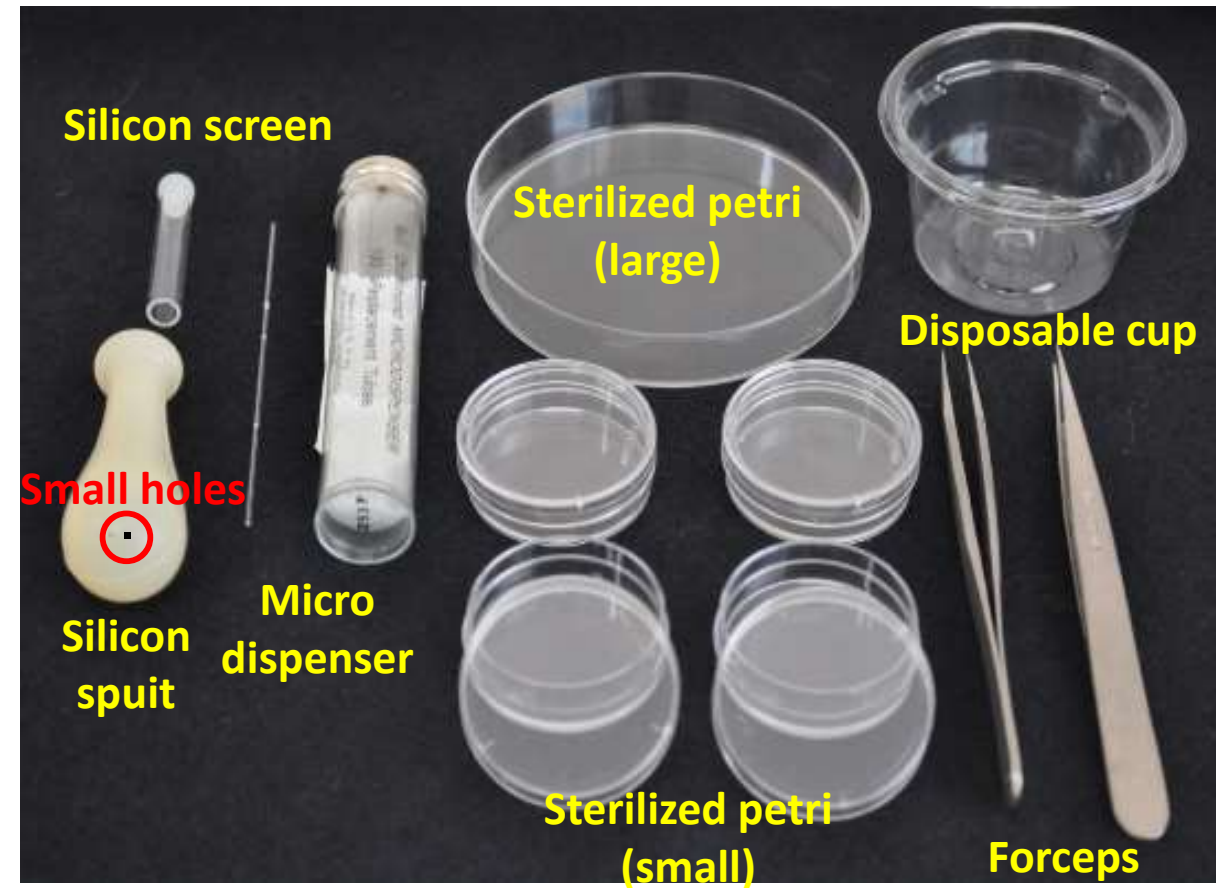


Thaw semen in water bath

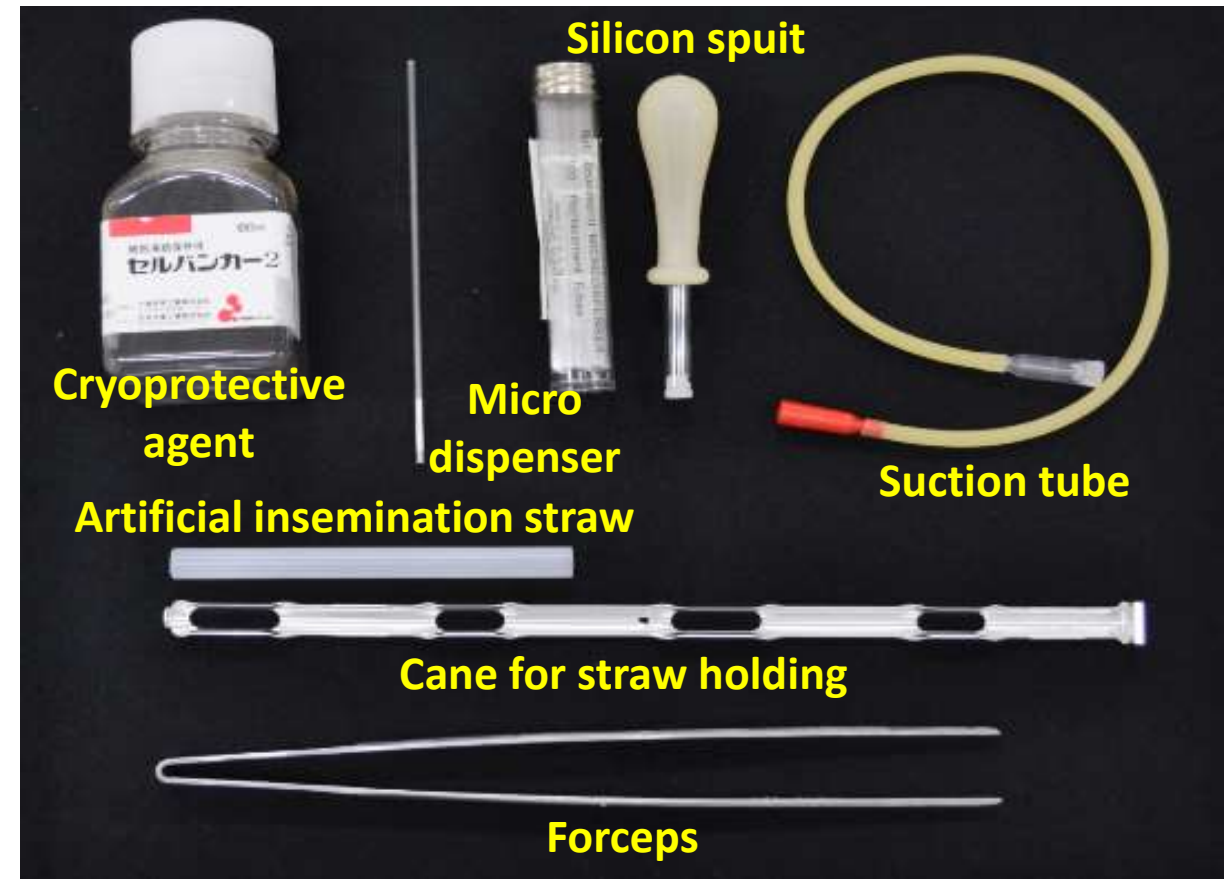


Inseminate female moth with the frozen and thawed semen

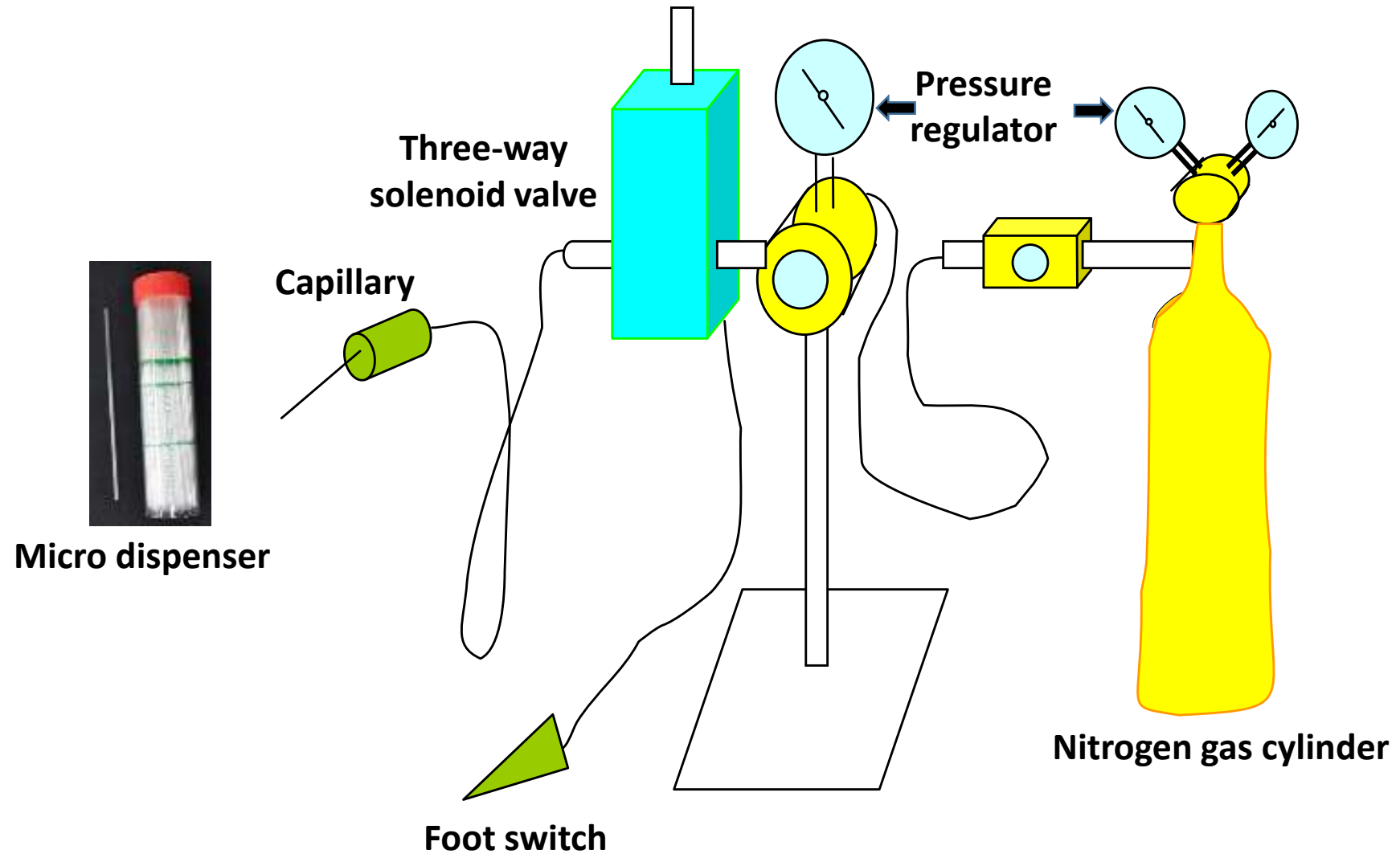
Procedure for cryopreserving the spermatozoa



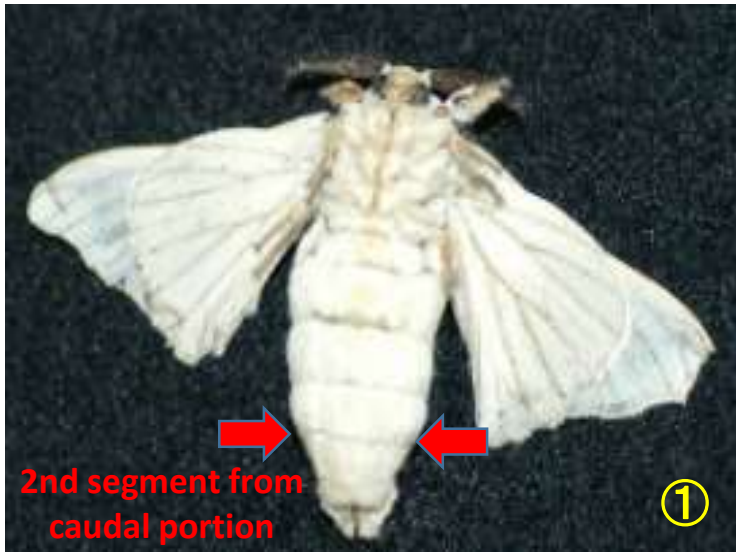
Lab wares used for collecting semen



Lab wares used for cryopreseving semen



Instrument for artificial insemination



2nd segment from caudal portion

①

Anchor the ventral part of the 2nd segment with forceps



②



③

Tear up the segment



Internal whole reproductive organs containing seminal vesicle

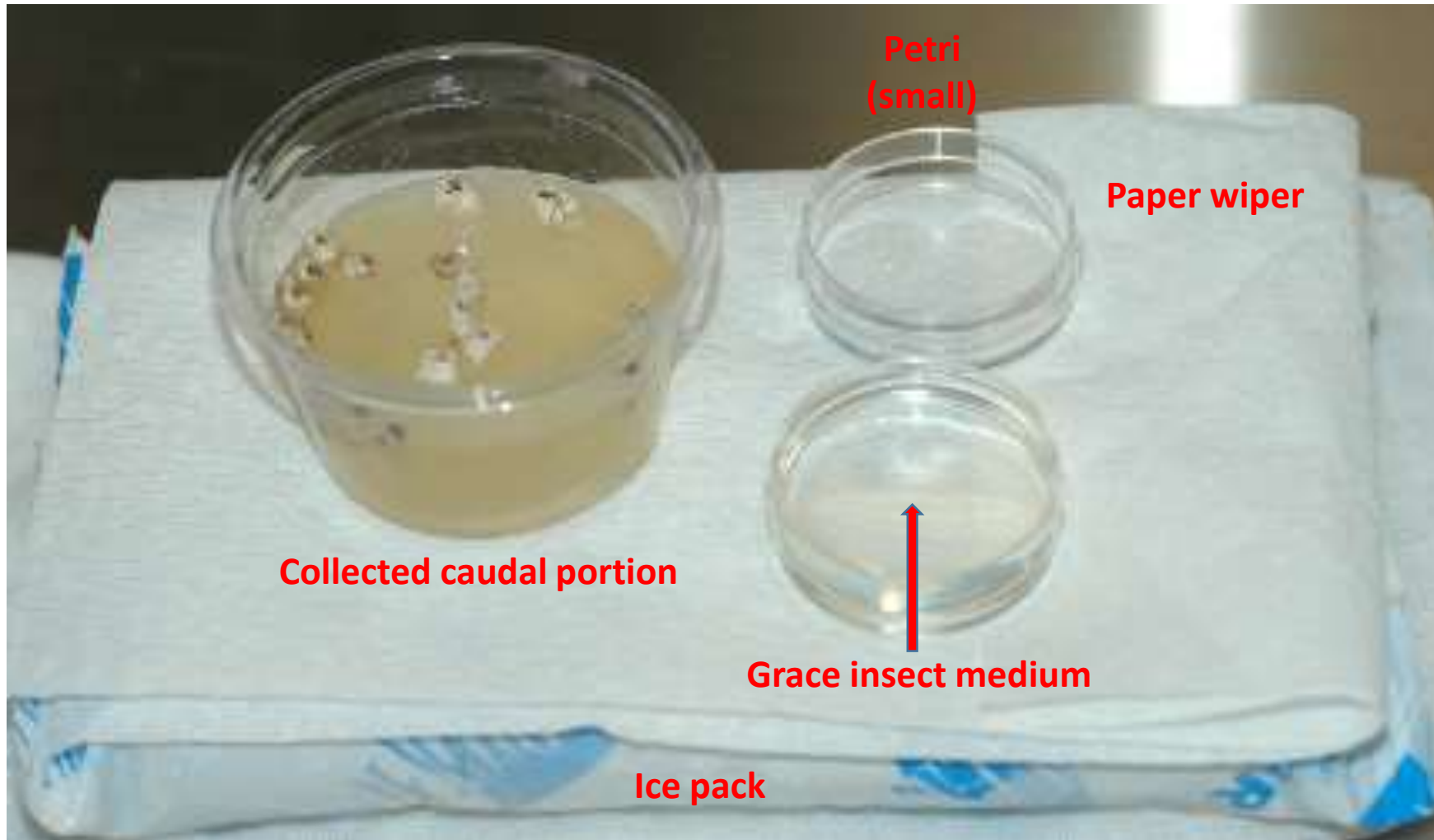
④



⑤

Caudal portion containing whole internal reproductive organs

Collect caudal portion containing whole internal reproductive organs



Prepare the collecting reproductive organs containing seminal vesicle



Fasten the innermost of the caudal portion softly with forceps



Hold the caudal portion down with the left forceps, and pull the reproductive organs softly with the right forceps



Cut the reproductive organs off at the tip of prostate gland

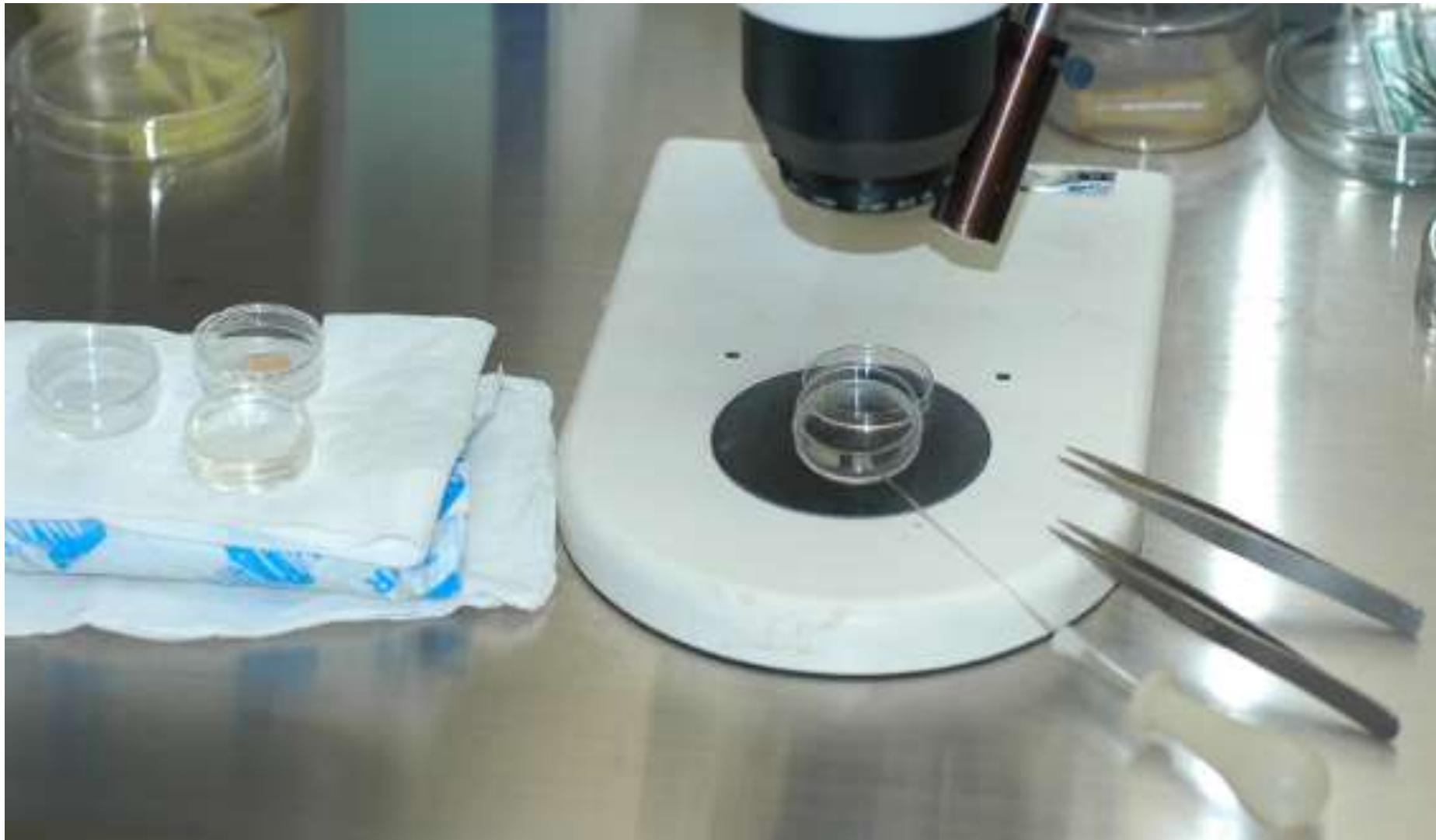
Collect the reproductive organs



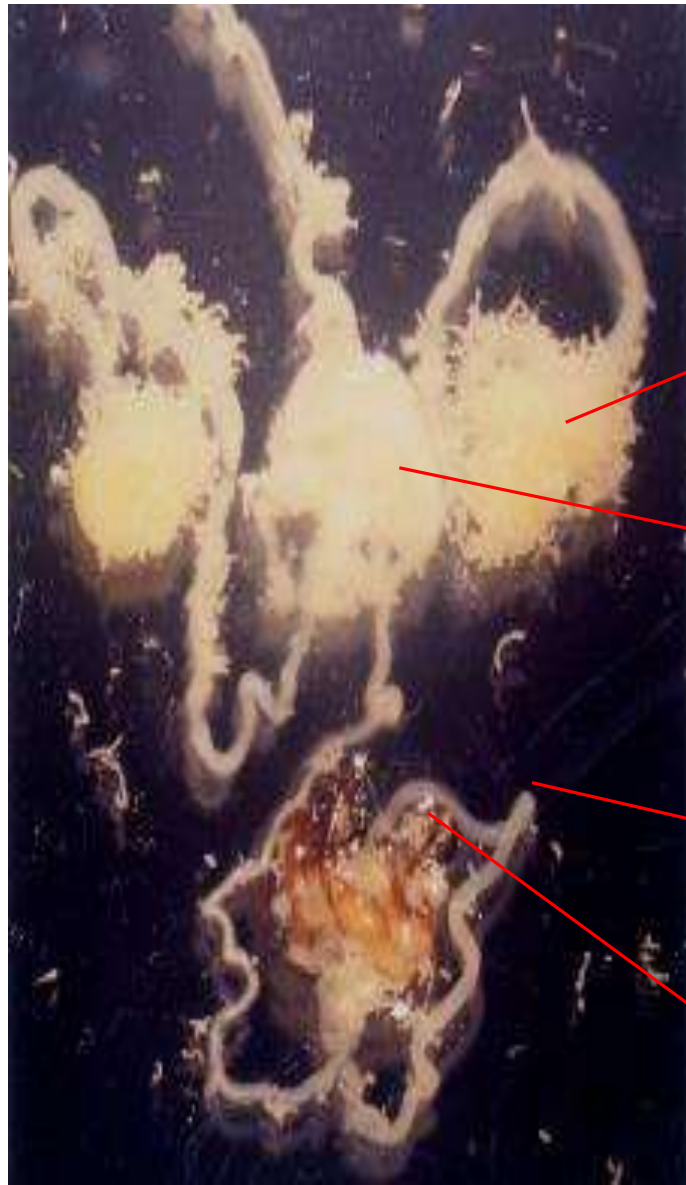
Collected reproductive organs

Wash the dirty things, like scaly hair, attached to the reproductive organs out in the petri filled with Grace insect medium

Wash the collected reproductive organs



Prepare the collecting semen

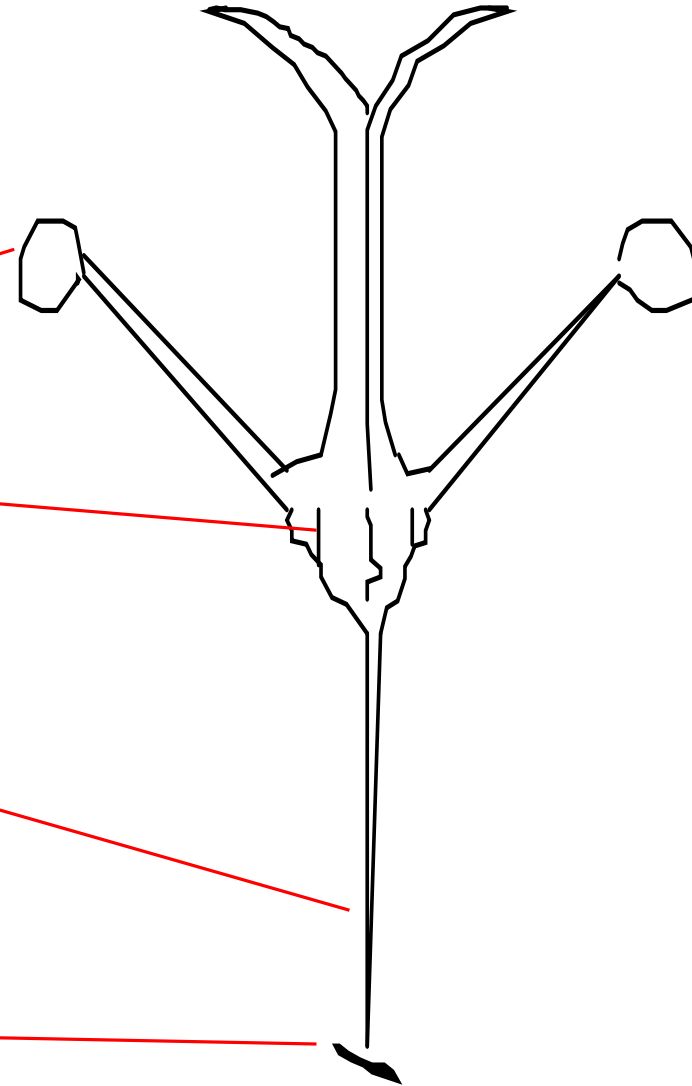


testis

seminal
vesicle
(containing
ampulla)

glandula
prostatica

penis



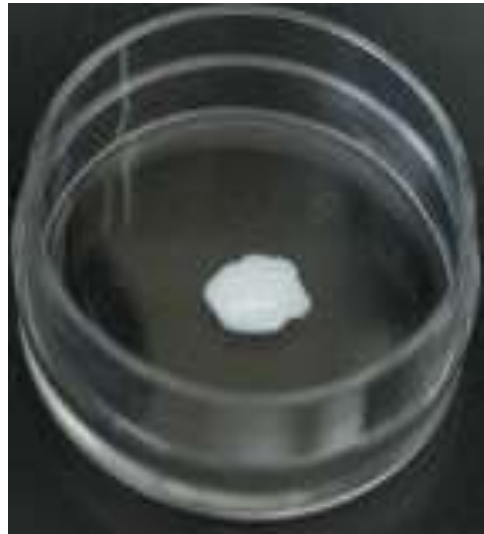
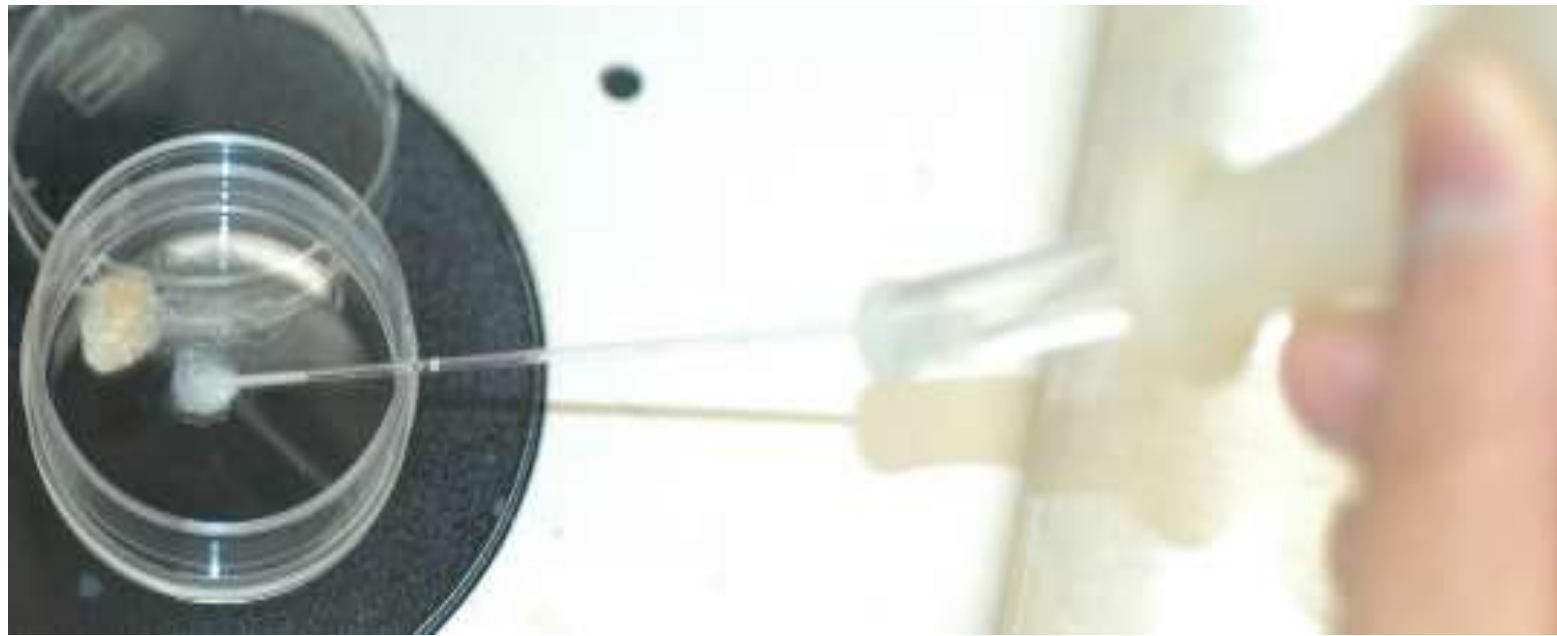
Reproductive organs of male moth



Spread the reproductive organs, and collect the semen by cutting the ampulla of the seminal vesicle off with forceps.

Cut the glandula prostatica off in the same manner to collect expressed prostatic secretion.

Collect the semen and expressed prostatic secretion



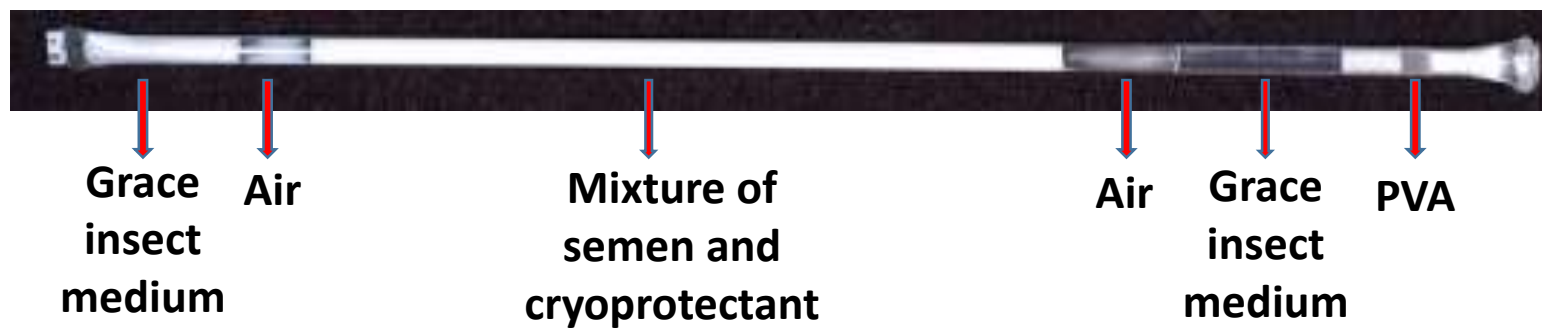
small holes

The pipette consist of micro dispenser,
silicon screen and silicon spuit

Collect the semen by using pipette



Store the semen in plastic artificial insemination straw by using suction tube



Store the semen in artificial insemination straw



Seal the both tips of the straw up with heat sealer



Freezer inside

Keep the straw in -80°C freezer for 10 min



Fill box of Styrofoam with liquid nitrogen. Then, place the straw frozen at -80°C in the liquid nitrogen



Then, attach the straw to the cane for straw holding in liquid nitrogen by using forceps (large-scale)



Store the cane in liquid nitrogen preserving container

Store the semen in liquid nitrogen



**Shift the straw into 37°C water bath
to thaw the semen**

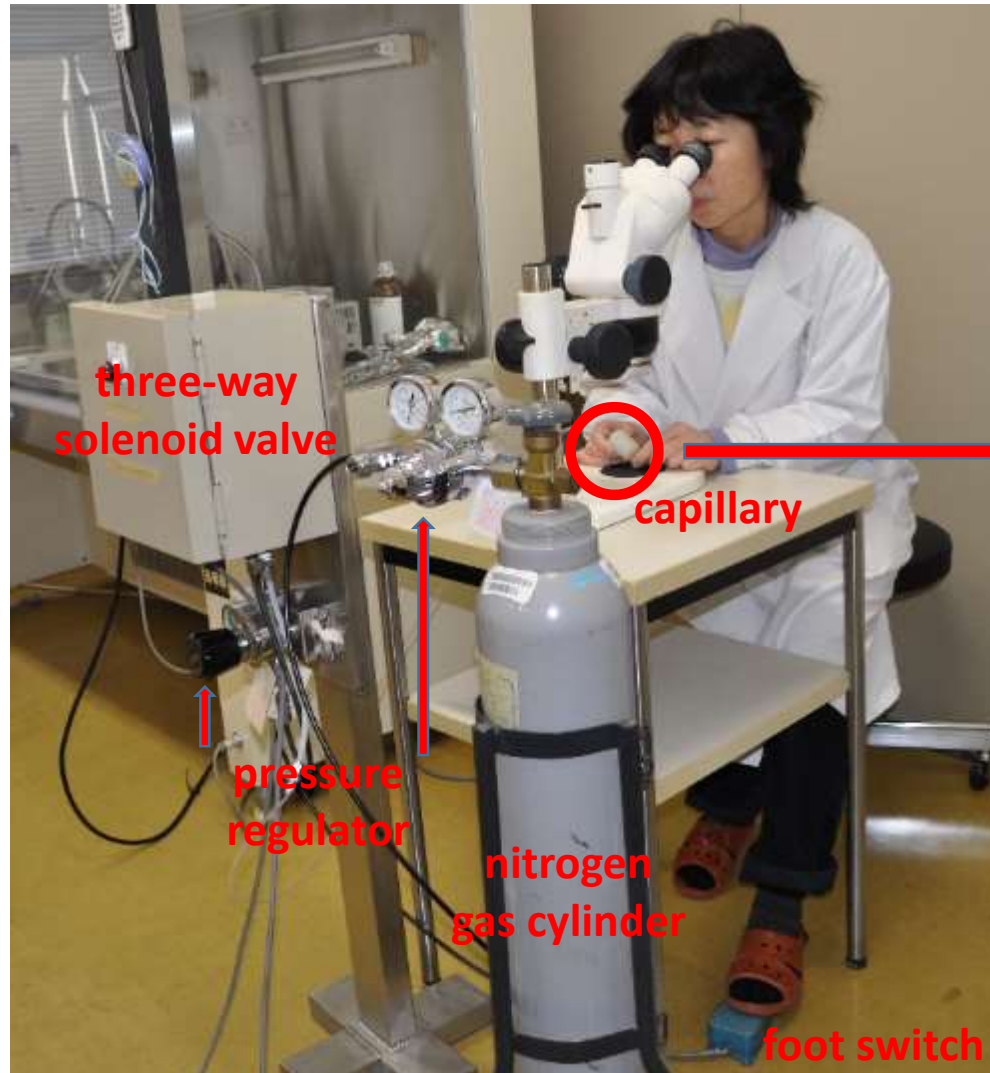


**Cut the straw at the portion containing
air, and collect the semen in petri**

Thaw the semen stored in liquid nitrogen



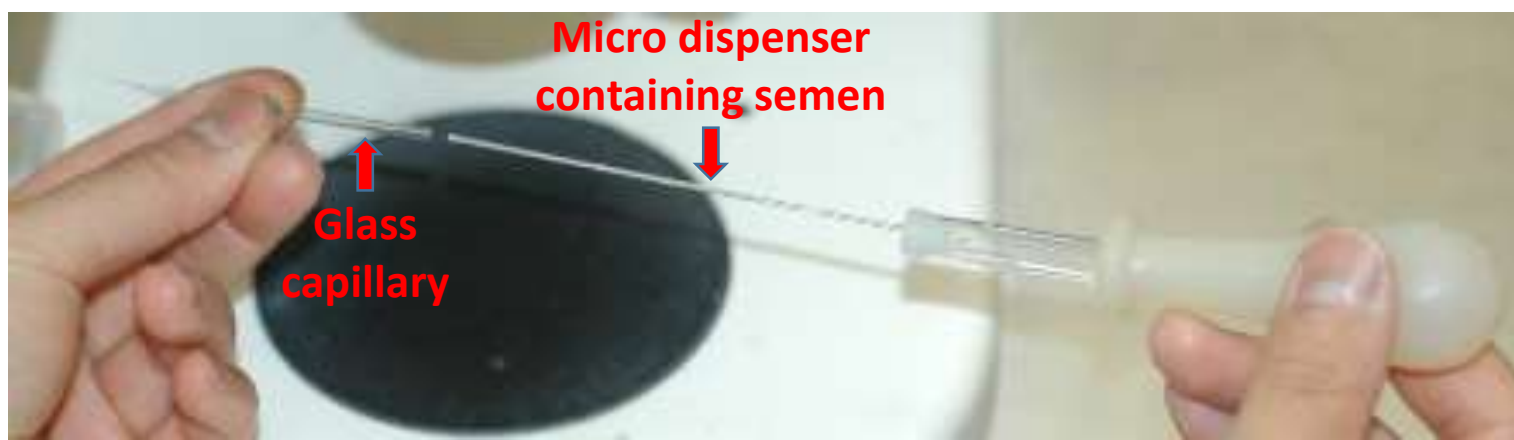
Mix the sperm with the trypsin solution at the time of artificial insemination



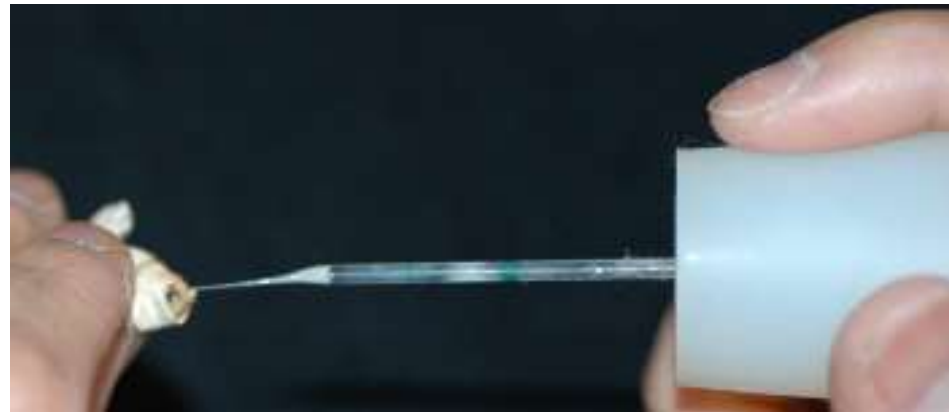
Artificial insemination work



Make the glass capillary



Load the semen into glass capillary

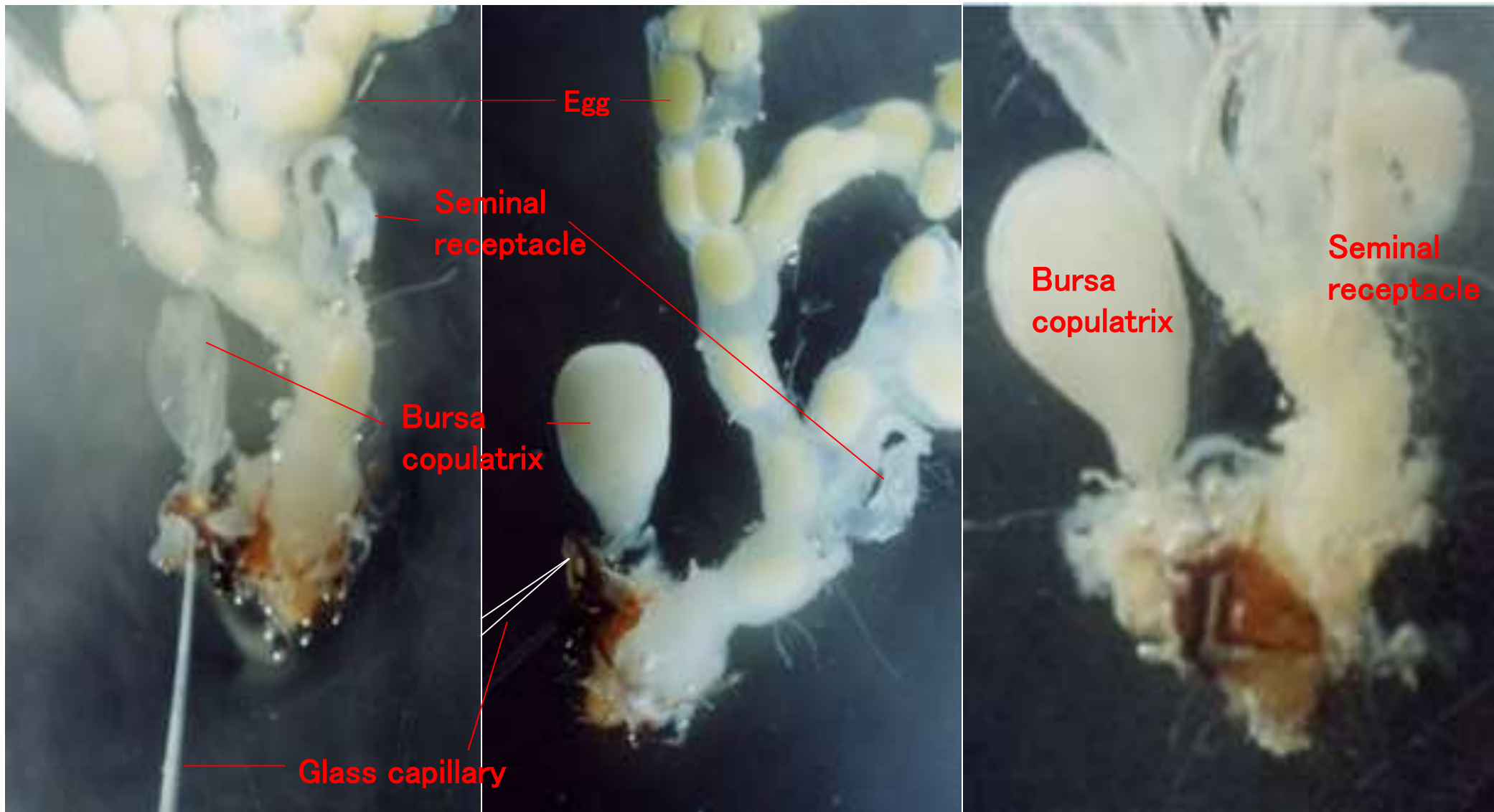


**Inject an aliquot of semen
into the bursa copulatrix
of virgin female**

Inject the semen into the bursa copulatrix



Manual operation for artificial insemination by using pipette

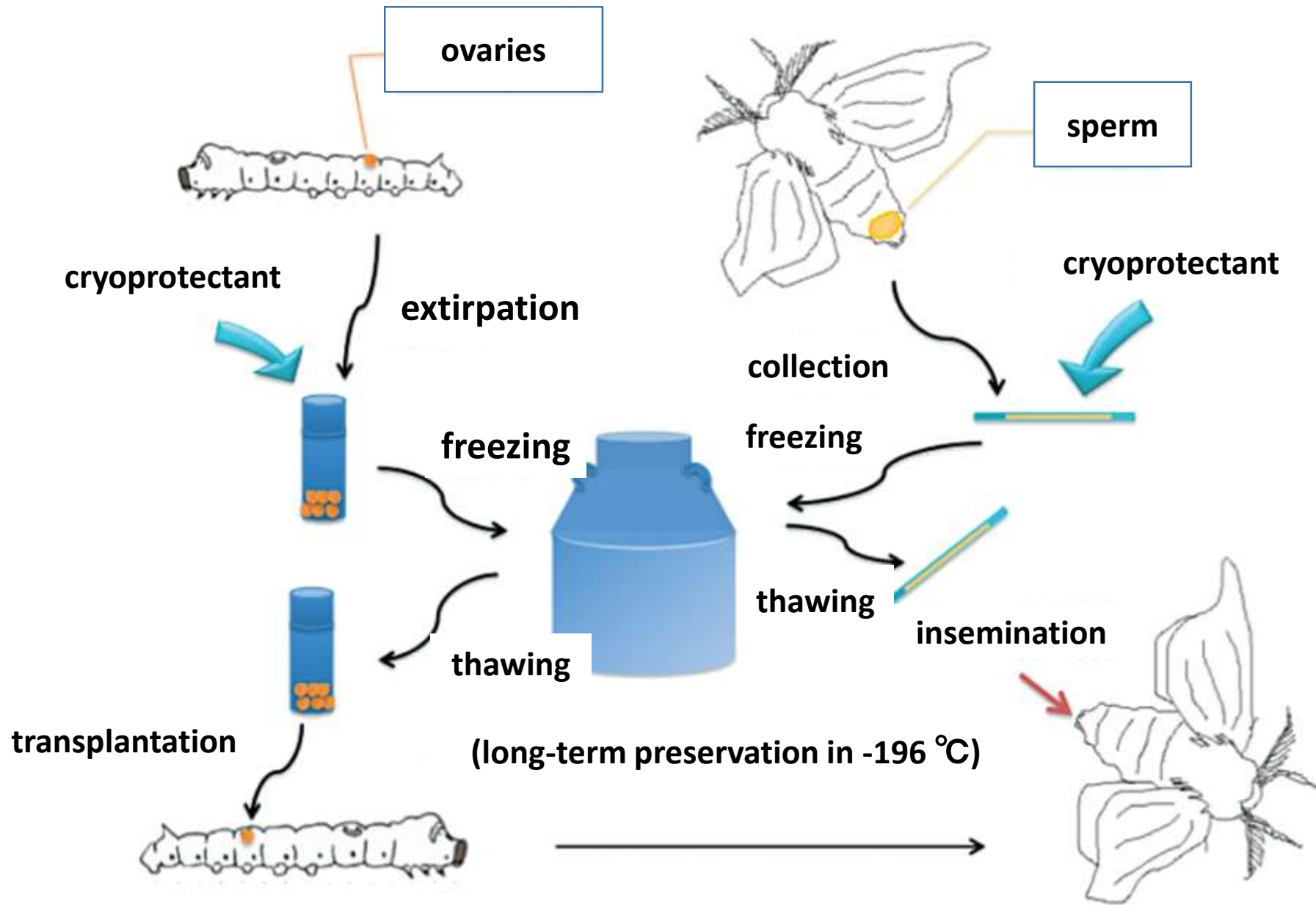


Before injection

After injection

After oviposition

Injection of semen into the bursa copulatrix of virgin female moth



Method for obtaining offspring using cryopreserved ovaries and sperm