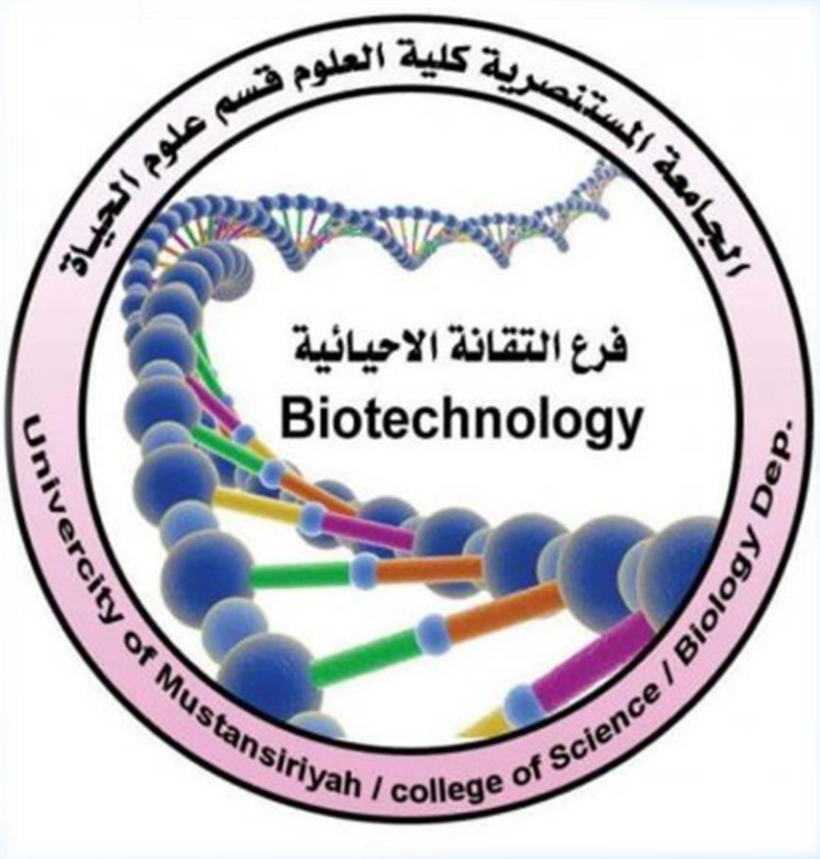
بسم الله الرحمن الرحيم

Embryonic stem cells production & maintenance

Prof. Dr. Sadeq Kaabi

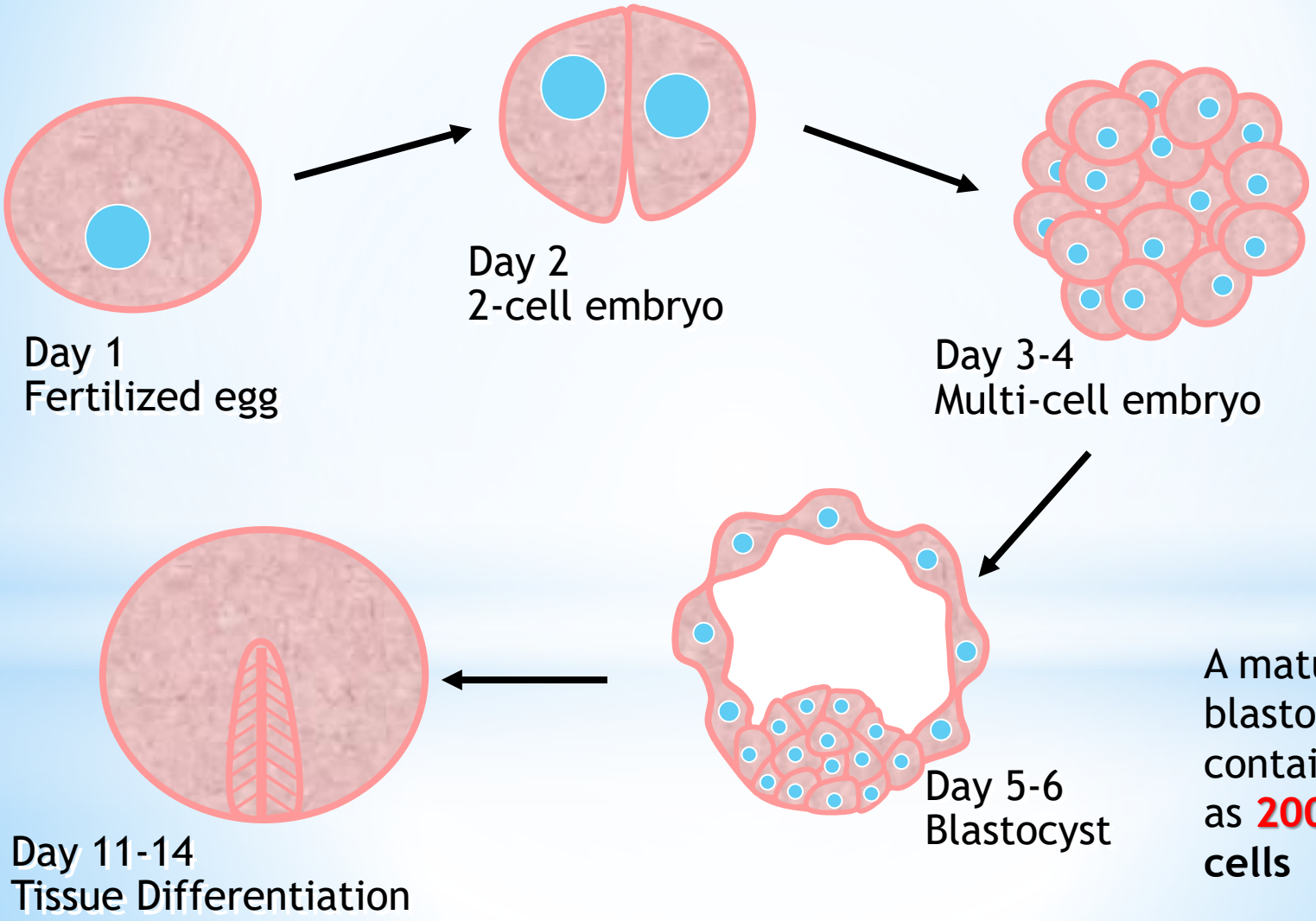


Ph.D. study
Biotechnology branch/ Biology Department
2024-2025

How are embryonic stem cells harvested?

- Growing cells in the laboratory is called as **cell culture**.
- Human ES cells are derived from 4-5 day old blastocyst
- Blastocyst structures include:
  - **Trophoblast**: outer layer of cells that surrounds the blastocyst & forms the placenta
  - **Blastocoel**: ("blastoseel") the hollow cavity inside the blastocyst that will form body cavity
  - **Inner cell mass**: a group of approx. **30 cells** at one end of the blastocoel:
    - Forms 3 germ layers that form all embryonic tissues (endoderm, mesoderm, ectoderm)

Stages of Embryogenesis

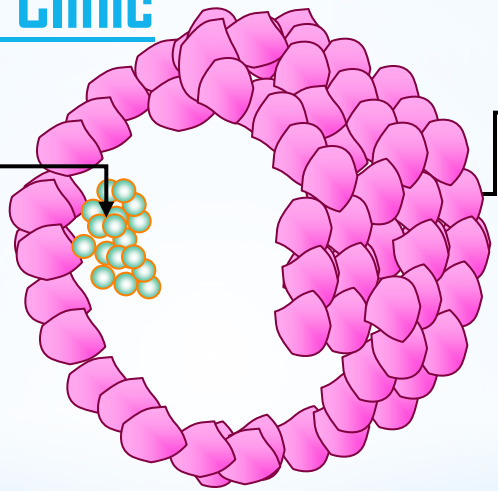


A mature blastocyst may contain as many as **200** to **300** cells

A primer on Human Embryonic Stem Cells

Blastocyst -

from In Vitro Fertilization Clinic

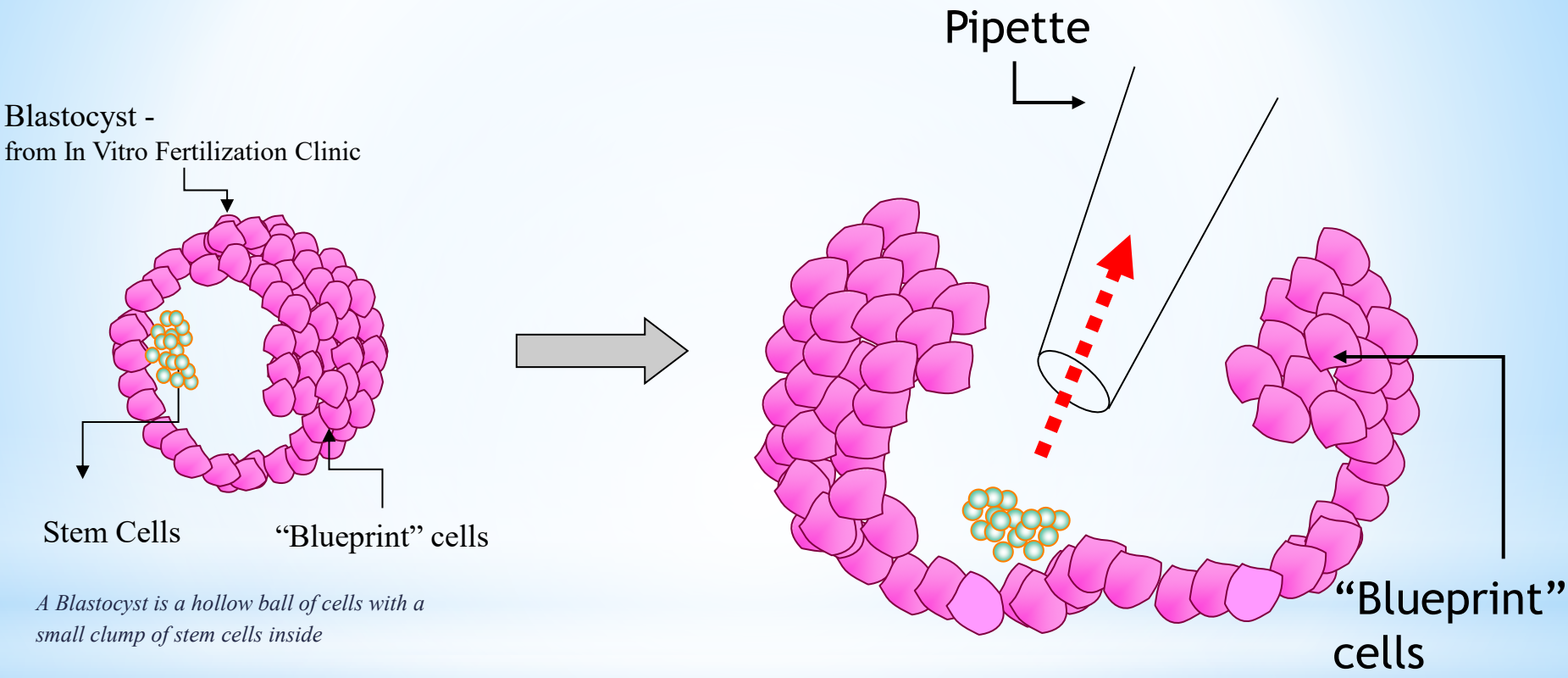


Inner Cell Mass
(Stem Cells)

"Blueprint" cells

A Blastocyst is a hollow ball of cells with a small clump of stem cells inside

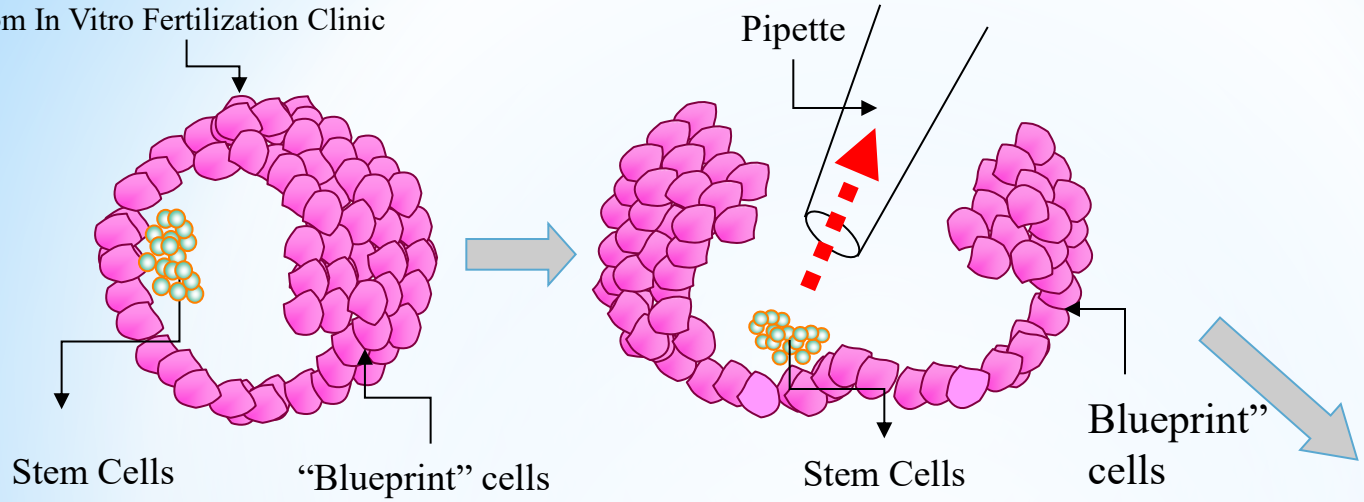
Human Embryonic Stem Cells



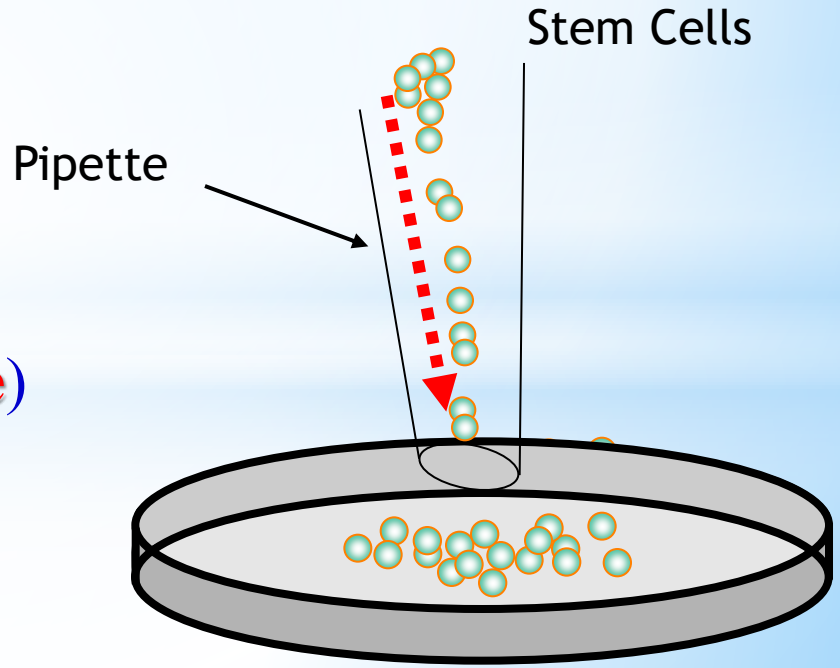
A Blastocyst is a hollow ball of cells with a small clump of stem cells inside

To remove the stem cells, the Blastocyst is opened and the stem cells removed with a pipette

Blastocyst -
from In Vitro Fertilization Clinic

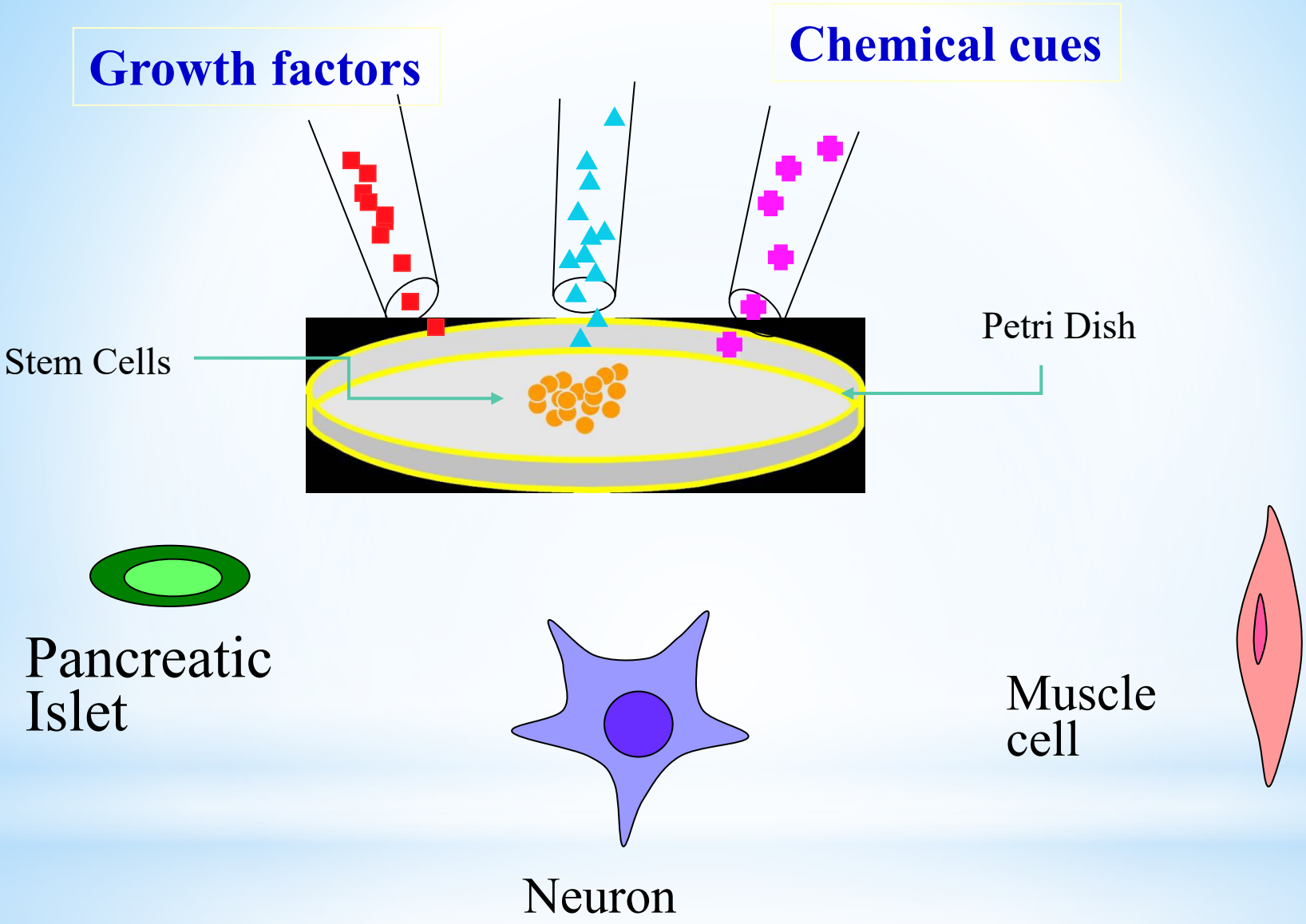


A Blastocyst is a hollow ball of cells with a small clump of stem cells inside



The stem cells are placed in a dish and are fed and cared for (**each blastocyst = 1 stem cell line**)

The area of an early blastocyst is $A \leq 18500\ \mu m^2$ with a mean diameter **d = 140 ± 9 µm**



Different chemicals / molecules are added to the stem cells to make them become specific types of cells.

Cell Culture Techniques for ESC

- Isolate & transfer of inner cell mass into plastic culture dish that contains culture medium

- Cells divide and spread over the dish

Inner surface of culture dish is typically coated with mouse embryonic skin cells that have been treated so they will not divide

- This coating is called a **FEEDER LAYER**

  - **Feeder cells provide ES cells with a sticky surface for attachment**
  - **Feeder cells release nutrients**

- **Recent discovery: methods for growing embryonic stem cells without mouse feeder cells**

►Over the course of several days the inner cell mass proliferate and begin to crowd the culture dish.

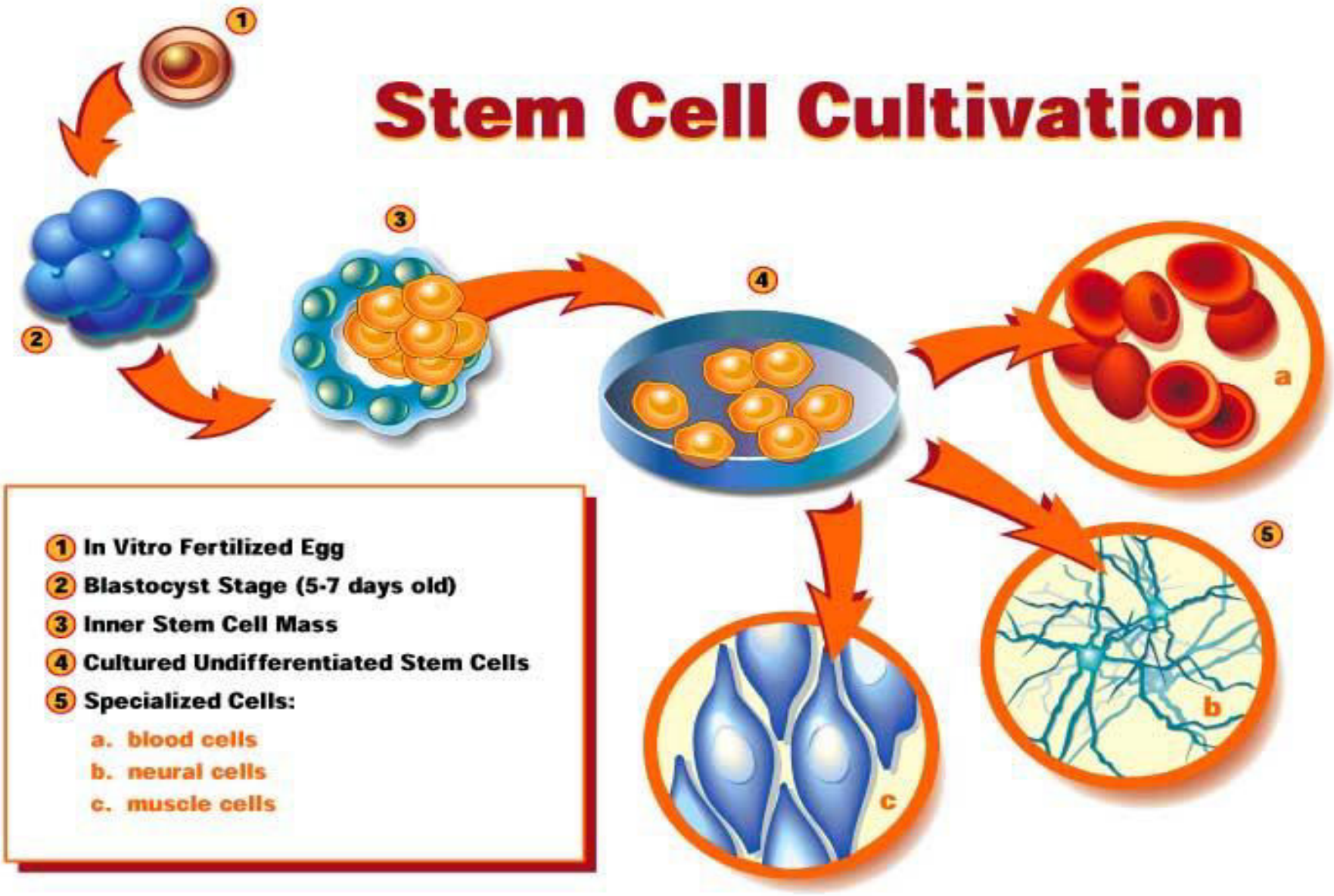
►They are then gently removed and plated into several fresh culture dishes.

The process of plating the cells is repeated several times and for many months and is called **subculturing** .

Each cycle of subculturing is referred to as a **passage**.

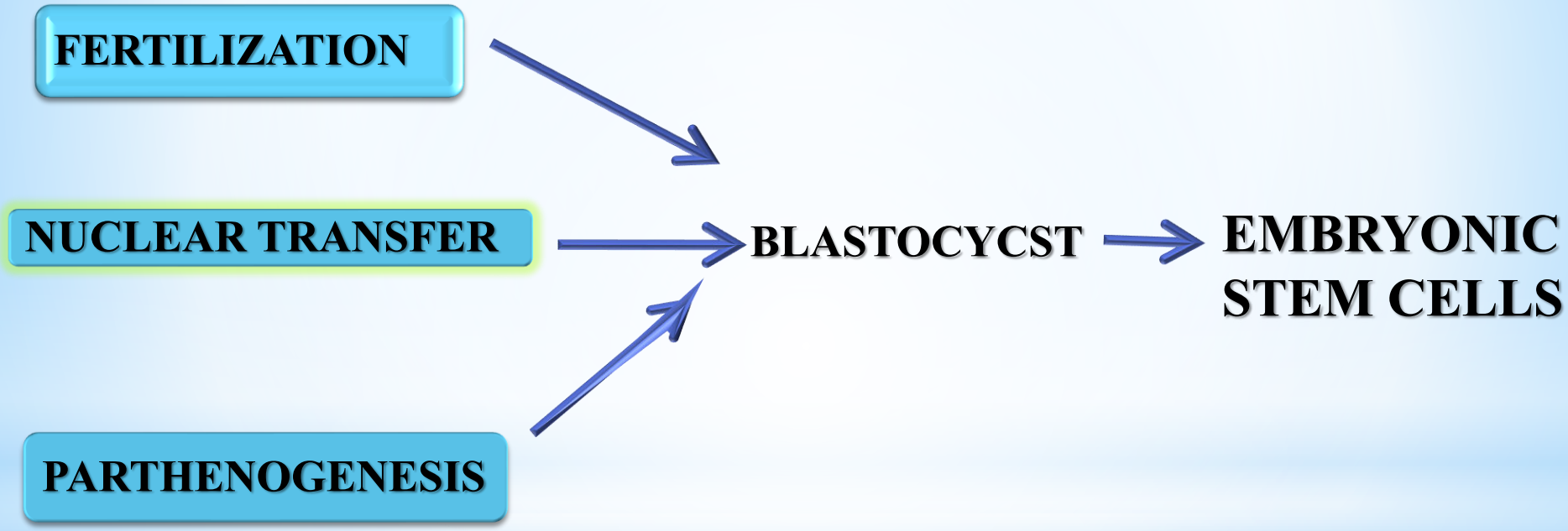
►**After six months or more the inner cell mass yield millions of embryonic stem cells**. These cells are Pluripotent and appear genetically normal and are referred to as an **embryonic stem cell line**.

Stem Cell Cultivation



1. In Vitro Fertilized Egg
2. Blastocyst Stage (5-7 days old)
3. Inner Stem Cell Mass
4. Cultured Undifferentiated Stem Cells
5. Specialized Cells:
   a. blood cells
   b. neural cells
   c. muscle cells

SOURCE OF EMBRYONIC STEM CELLS



DERIVATION OF EMBRYONIC STEM CELLS FROM FERTILIZED OOCYTE

OOCYTE
(ovaries of slaughtered animals, ovum pick-up)

↓

IVM

↓ ← **SPERM**

IVF

↓

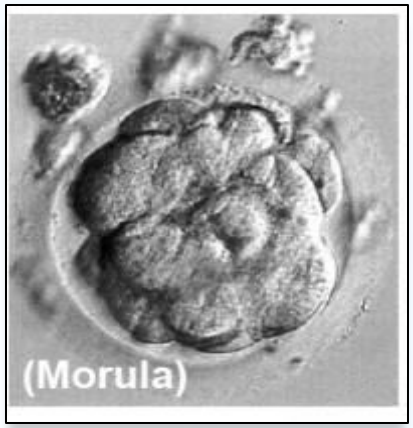
ZYGOTE

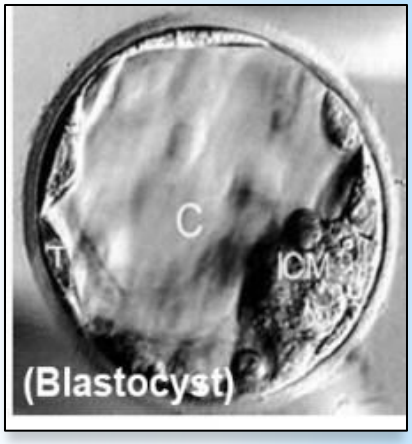
↓

MORULA
(16-32 cells) → **BLASTOCYST** → **ICM** → **Epiblast** → **Embryonic stem cells**

ICM → **Hypoblast**

BLASTOCYST → **TROPHOECTODERM**

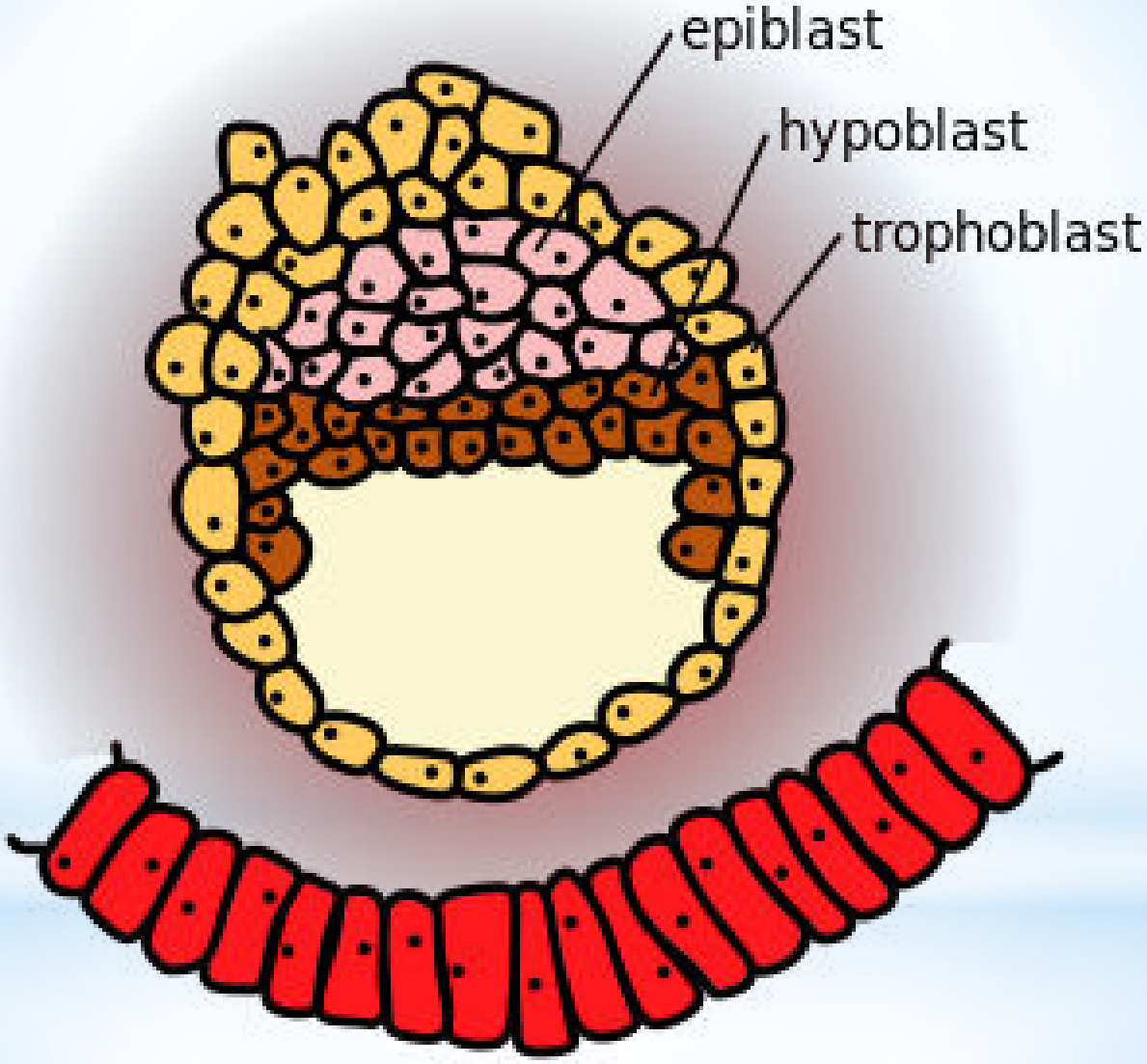




In vitro maturation (IVM) is when eggs are collected and matured outside the body. **This is done as part of an in vitro fertilization (IVF) procedure**. Eggs (also called oocytes) are formed inside a developing fetus' ovaries before birth. These eggs stay in a resting state in the ovaries until puberty, when normal hormonal changes cause an egg to mature (develop) and be released each month. During IVF, medications called "gonadotropins" result in more than one egg maturing at the same time. These eggs are then collected before they are released from the ovary and are then placed with sperm in the laboratory in hopes of fertilization. In some cases, some or all of the oocytes (eggs) that are collected are not mature and ready to be fertilized. In years past, these eggs were not able to be used for IVF. Advances in science are beginning to allow embryologists to take these immature eggs and sometimes "ripen" them in vitro (in the laboratory). This is called IVM.

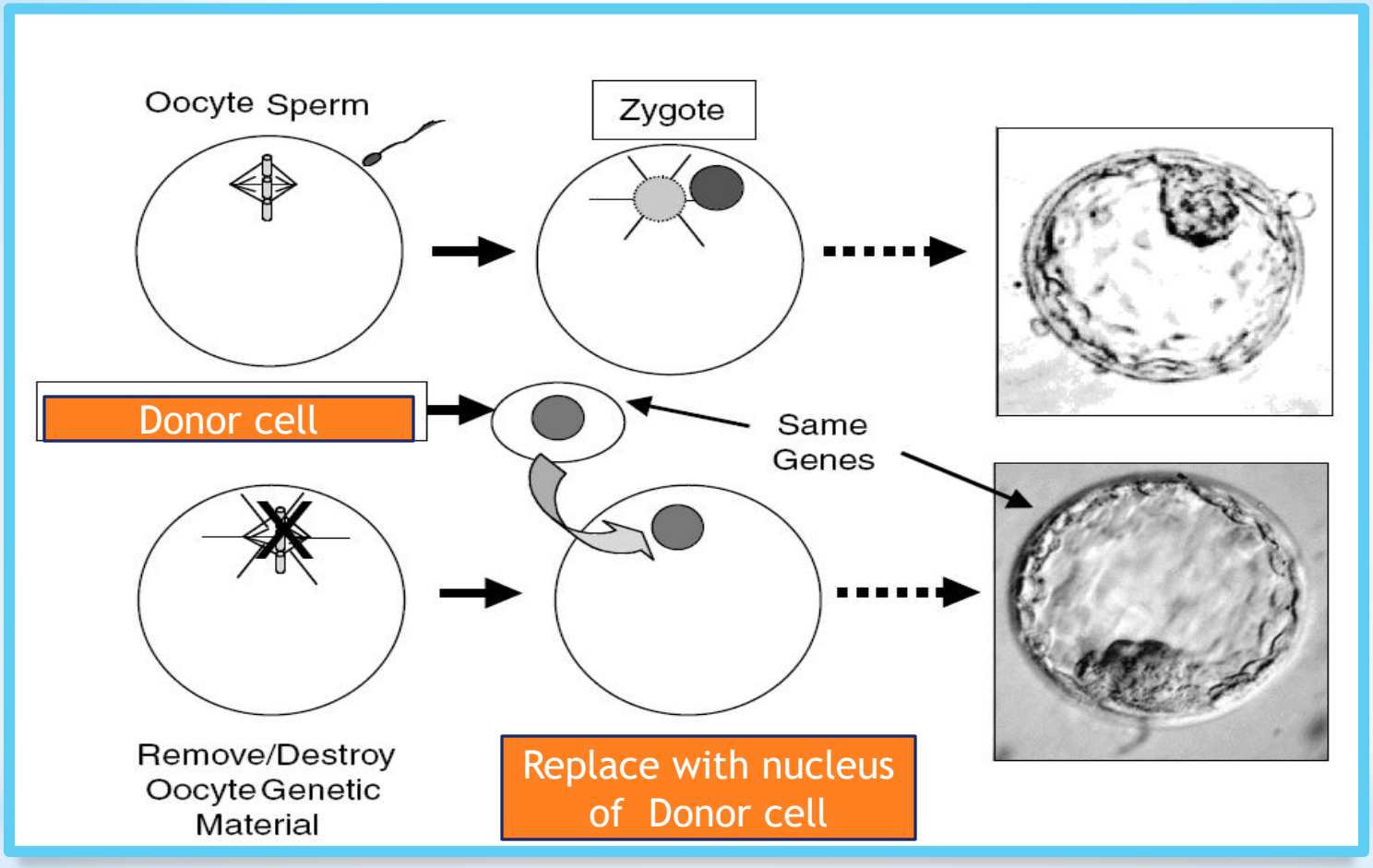
What is the IVM process?

Step 1: Baseline Testing. On day 2 of your cycle, you will have an ultrasound and a blood test.
Step 2: Hormone Stimulation. ...
Step 3: **Trans Vaginal Oocyte Aspiration** (**TVOA**) ...
Step 4: **Egg Culture**. ...
Step 5: (**Intracytoplasmic Sperm Injection** (**ICSI**)) ...
Step 6: Embryo Culture.



Human Embryo Day9

CELLS DERIVED FROM NUCLEAR TRANSFER



- In NT, the genetic material of the oocyte is removed and replaced with a diploid nucleus from a somatic (body) cell.
- This divides to yield an NT blastocyst whose genes are identical with those of the donor somatic cell.
- NT blastocysts, like normal blastocysts, can be used to derive embryonic stem cells from their inner cell masses.

CELLS DERIVED FROM PARTHENOGENESIS

IVM oocyte

↓

Calcium ionophore (5µM;5min)

↓

6-DMAP (4hr)

↓

IVC (in vitro culture)

↓

Blastocyst

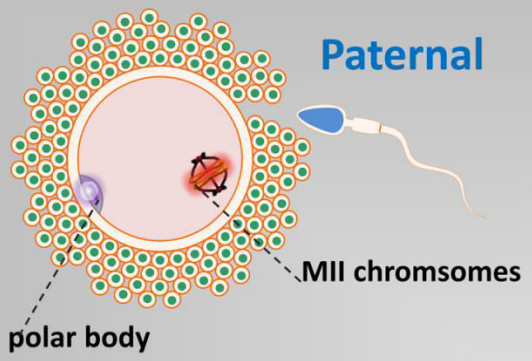
↓

ICM (inner cell mass)

↓

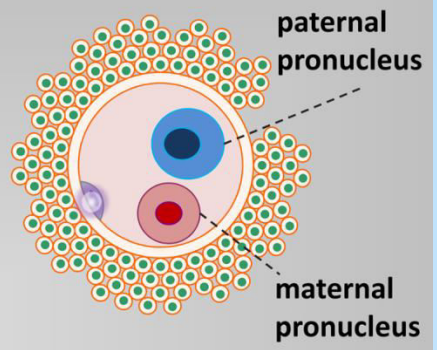
Embryonic stem cells

Artificial stimulation of oocyte by Ca^{2+} ionophore



Maternal

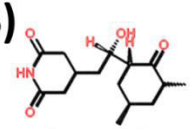
- Natural mating
- IVF

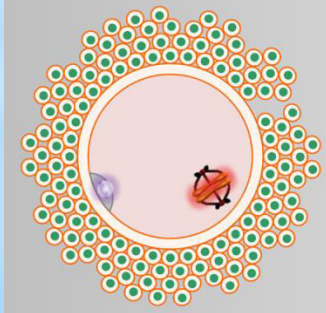


Zygote, biparental
- 1 maternal,
- 1 paternal genome

(1) Artificial stimulation:
- Ca^{2+} ionophore **(ionomicin)**
- electrical activation
- microinjection of Ca^{2+} into cytoplasm

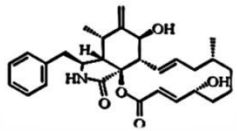
(2) Inhibition of MPF (cyclin B)
- cycloheximide

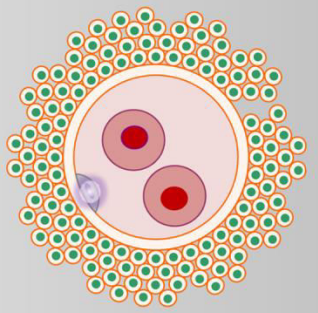




Maternal

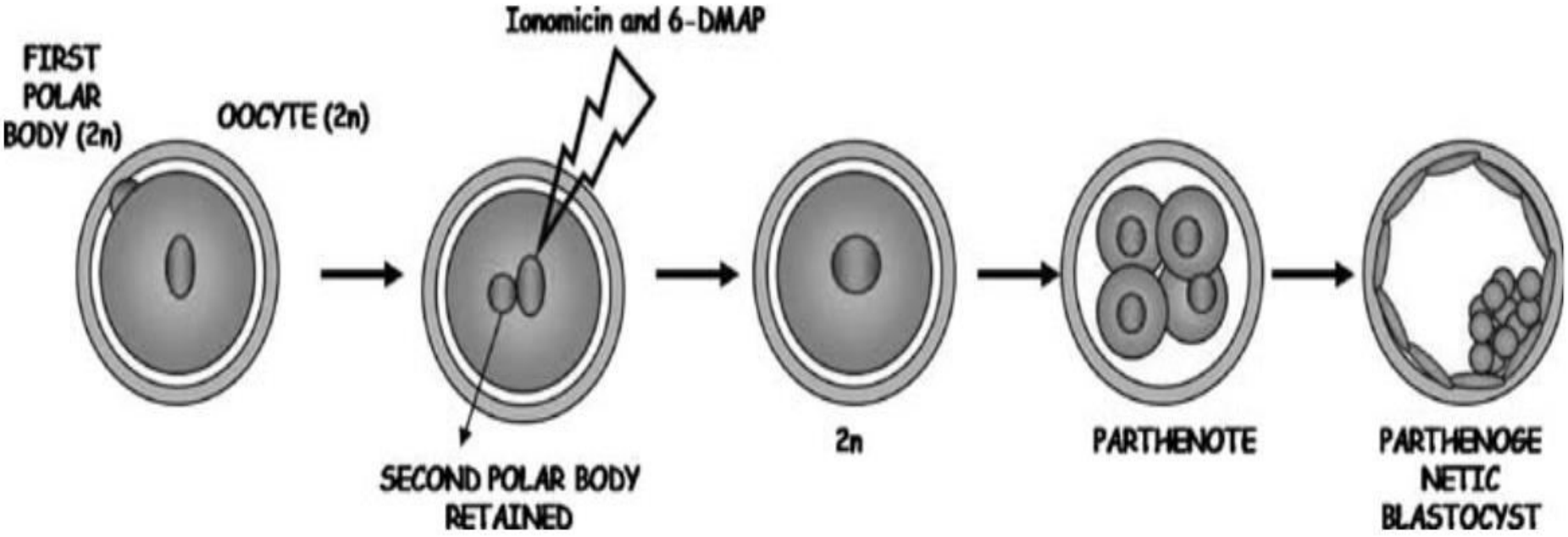
(2) Blockade of 2nd polar body extrusion
- cytochalasin B

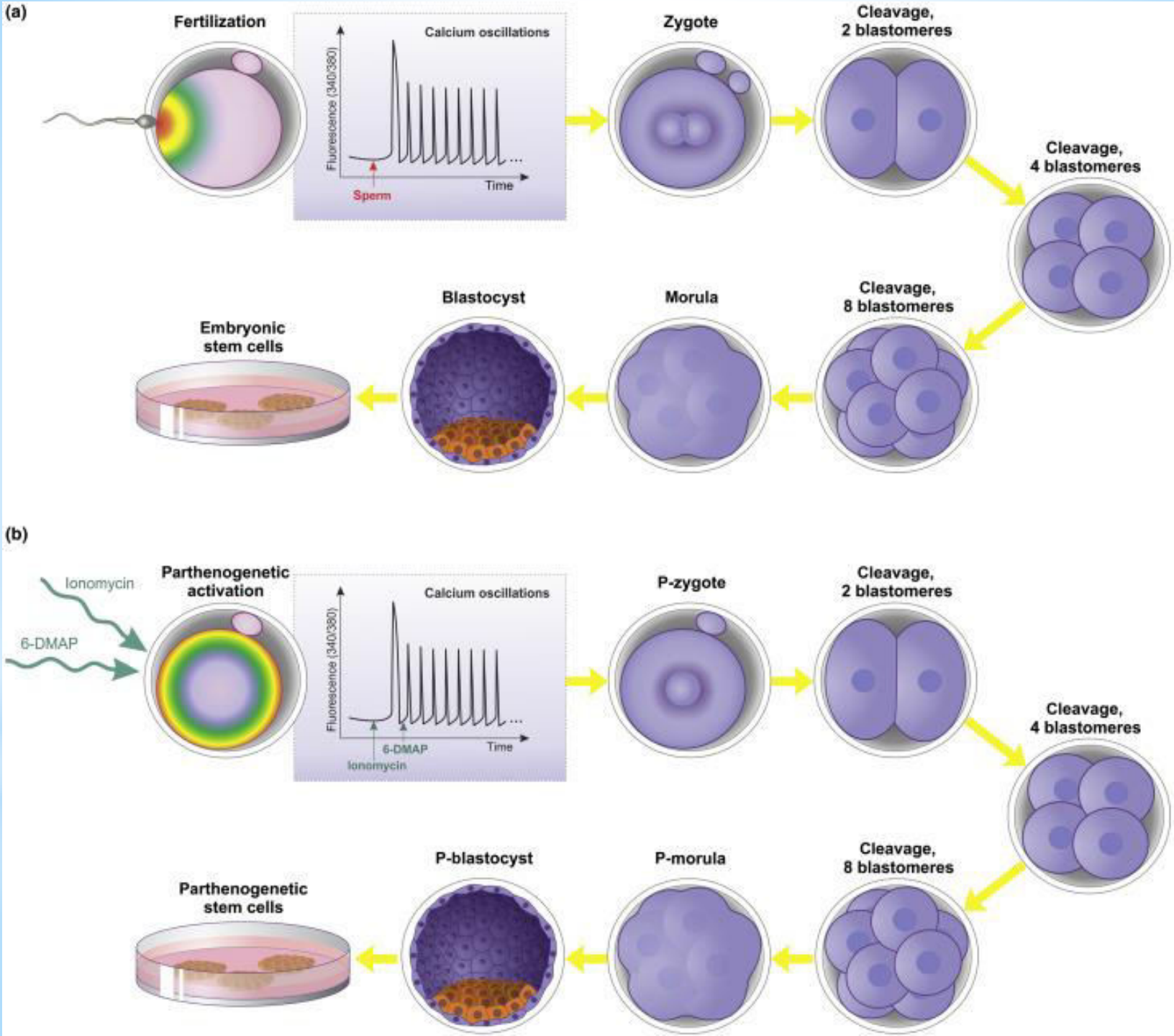




Parthenote, uniparental
(2 maternal genomes)

Schematic illustration of in vitro **parthenogenetic activation** of human oocytes, using **ionomicin** and **6-DMAP**. **The increase in intracellular calcium, induced by the exposure to ionomicin, mimics the biochemical events normally caused by sperm penetration and drives the oocyte into its developmental program**. **The use of adequate concentration of 6-DMAP prevents the extrusion of the polar body at meiotic divisions.**



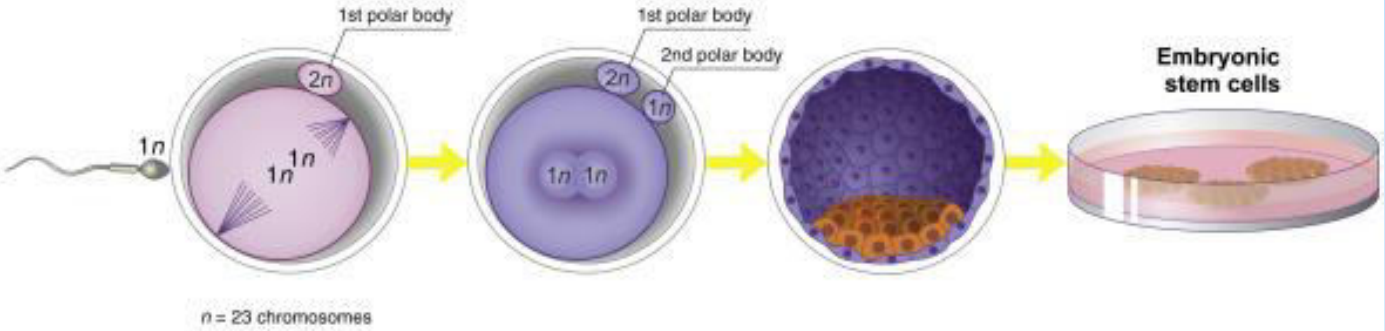

(a)
Fertilization
Calcium oscillations
Fluorescence (340/380)
Time
Sperm
Zygote
Cleavage, 2 blastomeres
Cleavage, 4 blastomeres
Cleavage, 8 blastomeres
Morula
Blastocyst
Embryonic stem cells
(b)
Ionomycin
6-DMAP
Parthenogenetic activation
Calcium oscillations
Fluorescence (340/380)
Time
6-DMAP
Ionomycin
P-zygote
Cleavage, 2 blastomeres
Cleavage, 4 blastomeres
Cleavage, 8 blastomeres
P-morula
P-blastocyst
Parthenogenetic stem cells

Parthenogenetic Activation of Unfertilized Oocytes

Oocyte activation can be induced by a variety of stimuli including electrical, chemical, and mechanical stimuli, and even spontaneous activation can happen. With the exception of two hpSC lines from spontaneously activated oocytes, all other reported hpSC lines originated from oocytes stimulated by chemical agents. (Other lines may have been derived in industry settings or not reported.) The chemicals used are chosen because they initiate transients in calcium concentration in the egg cytoplasm similar to sperm-induced repetitive Ca^{2+} oscillations (also called calcium waves) that persist for several hours after spermatozoon penetration. These **calcium waves** are critical for moving the egg out of meiotic arrest and into further development (Figures 1(a) and 2(a)).

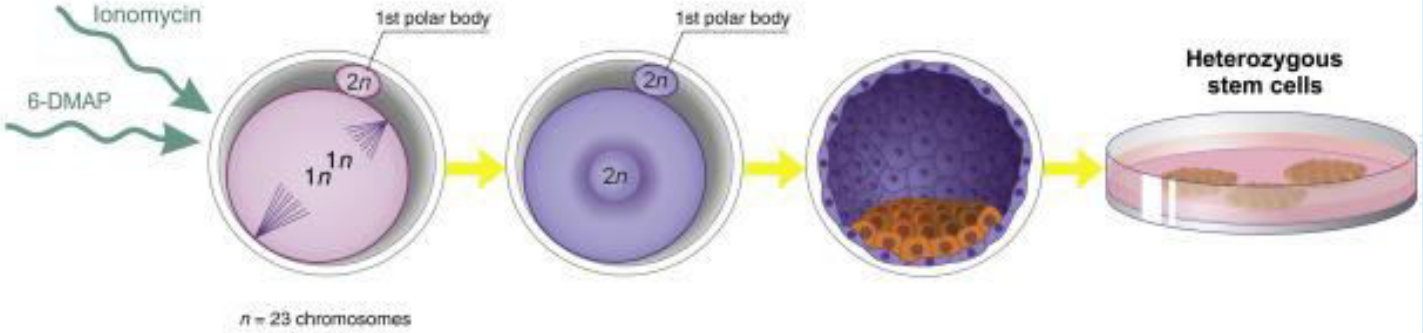
(a)

Fertilization



(b)

Parthenogenetic activation: heterozygous stem cells



(c)

Parthenogenetic activation: homozygous stem cells

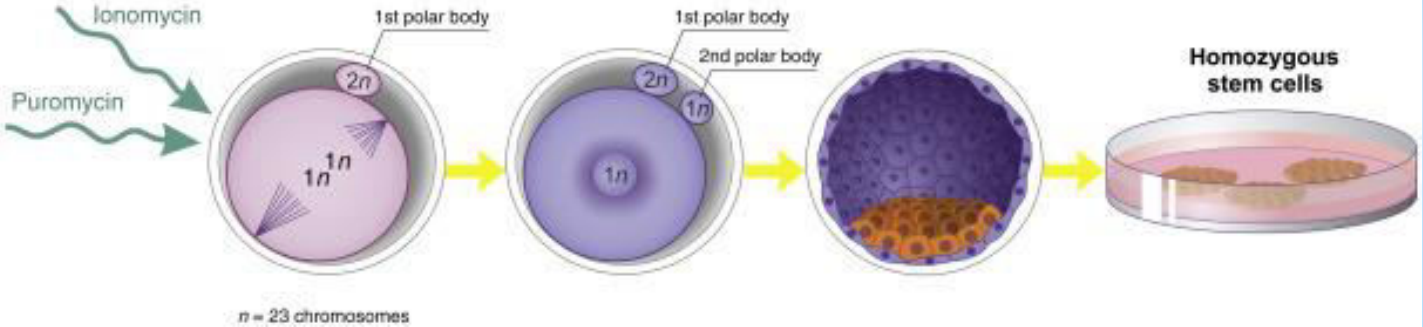
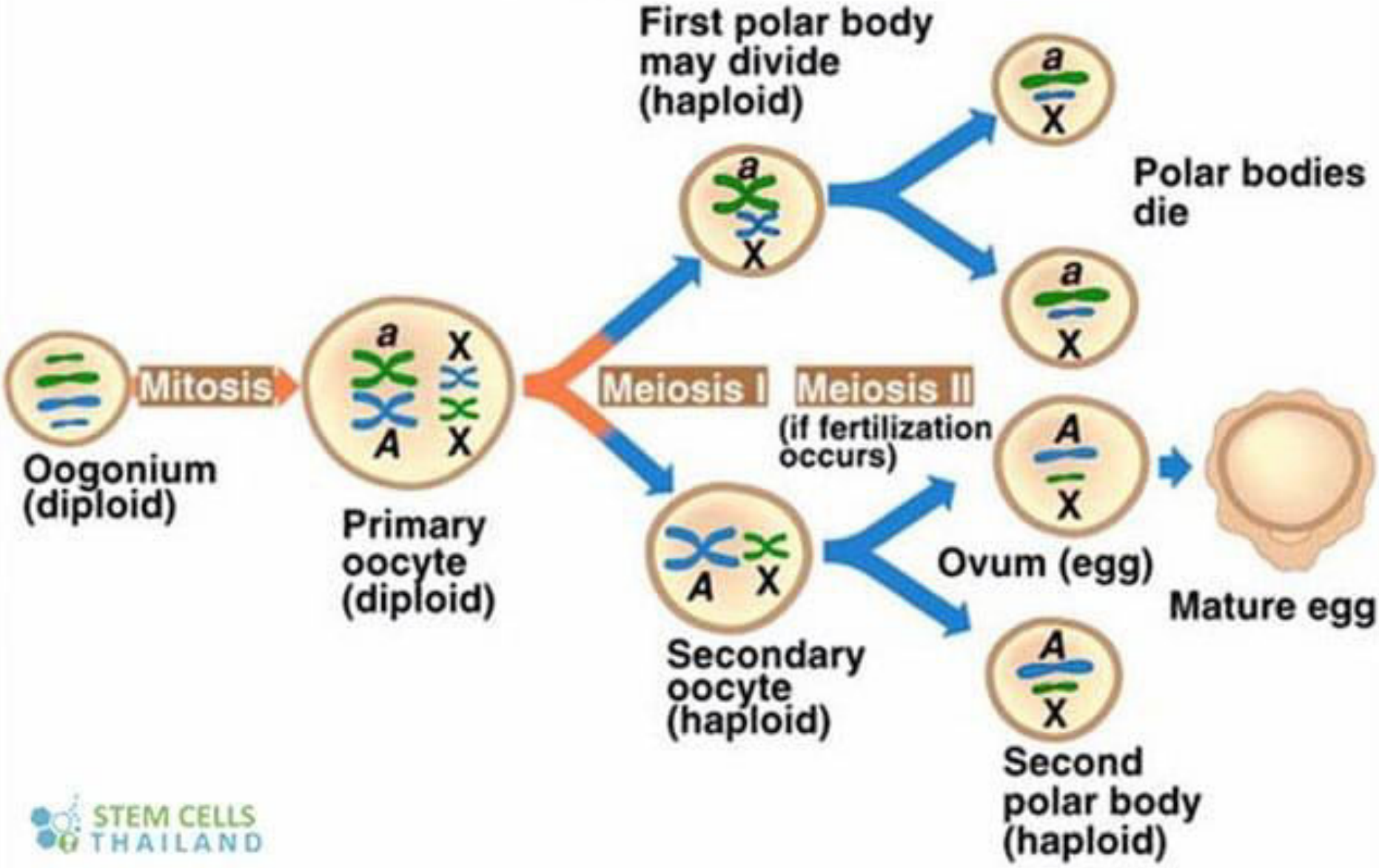


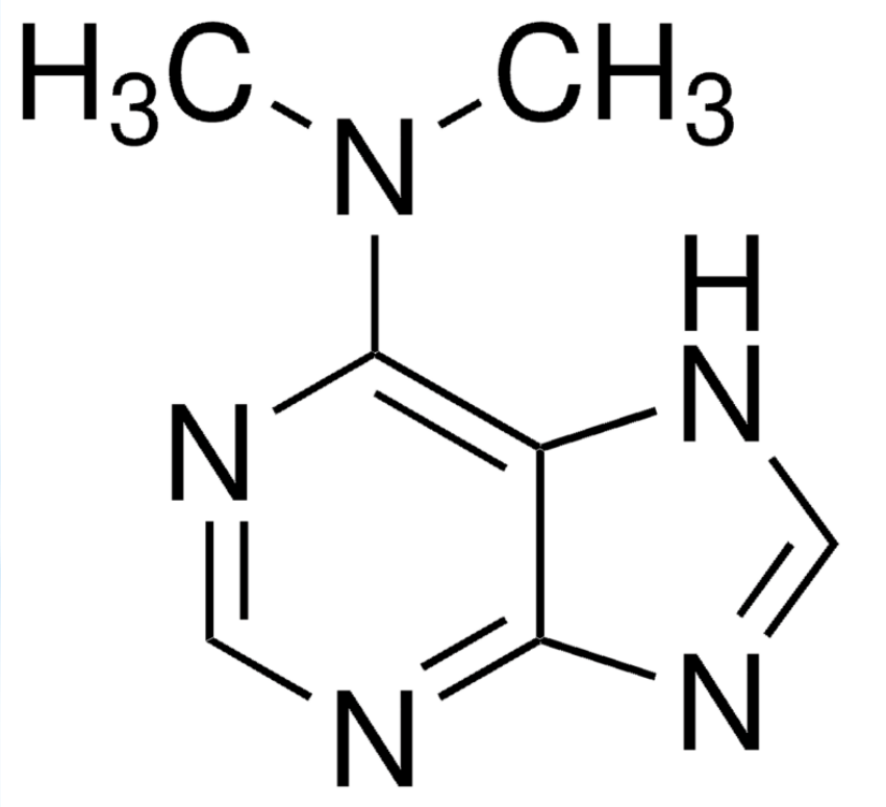
Figure 1. Parthenogenetic activation simulates spermatozoon-caused calcium waves in oocyte. (a) The penetration of human spermatozoon causes calcium oscillations that are part of signal releasing oocyte from meiotic arrest and permitting embryonic development (oocyte activation). The graph represents time-dependent fluctuations of intracellular free calcium. Spermatozoon-activated human oocyte follows development program through release of second polar body and formation of zygote with male and female pronuclei, subsequent passing the cleavage from two to eight blastomeres, formation of morula and blastocyst. (b) Similar to fertilization, parthenogenetic activation causes calcium oscillations in oocyte (graph represents tendency and not actual oscillations): **short-time ionomycin treatment simulates first large calcium oscillations and long-term 6-DMAP treatment supports prolonged calcium waves**. Parthenogenetically activated human oocyte follows development program similar to fertilized oocyte. Human parthenogenetic stem cells can be isolated from p-blastocyst. P-zygote, p-morula, and p-blastocyst are the structures that are similar to human zygote, morula, and blastocyst (developed from fertilized oocyte) but originated from parthenogenetically activated human oocyte.

Oogenesis



STEM CELLS THAILAND

6-Dimethylaminopurine
6- DMAP



METHODS OF ISOLATION

- **Immunosurgery**
- **Enzymatic**
- **Mechanical**
- **Intact blastocyst culture**

IMMUNOSURGERY

- Blastocyst is **treated with pronase to dissolve the zona pellucida**
- **Preincubated with antiserum** (rabbit anti-mouse serum)
- **Exposure to complement** (Guinea-pig complement serum)
- **Resulting cytotoxicity selectively kills trophoblasts**
- Large number of ICMs can be isolated simultaneously
- May cause cytotoxicity to ICM also
- **Protocol work best with high quality embryos containing intact trophoectoderm**, as only **structural integrity of blastocyst** prevents ICM from being susceptible to immunological reaction

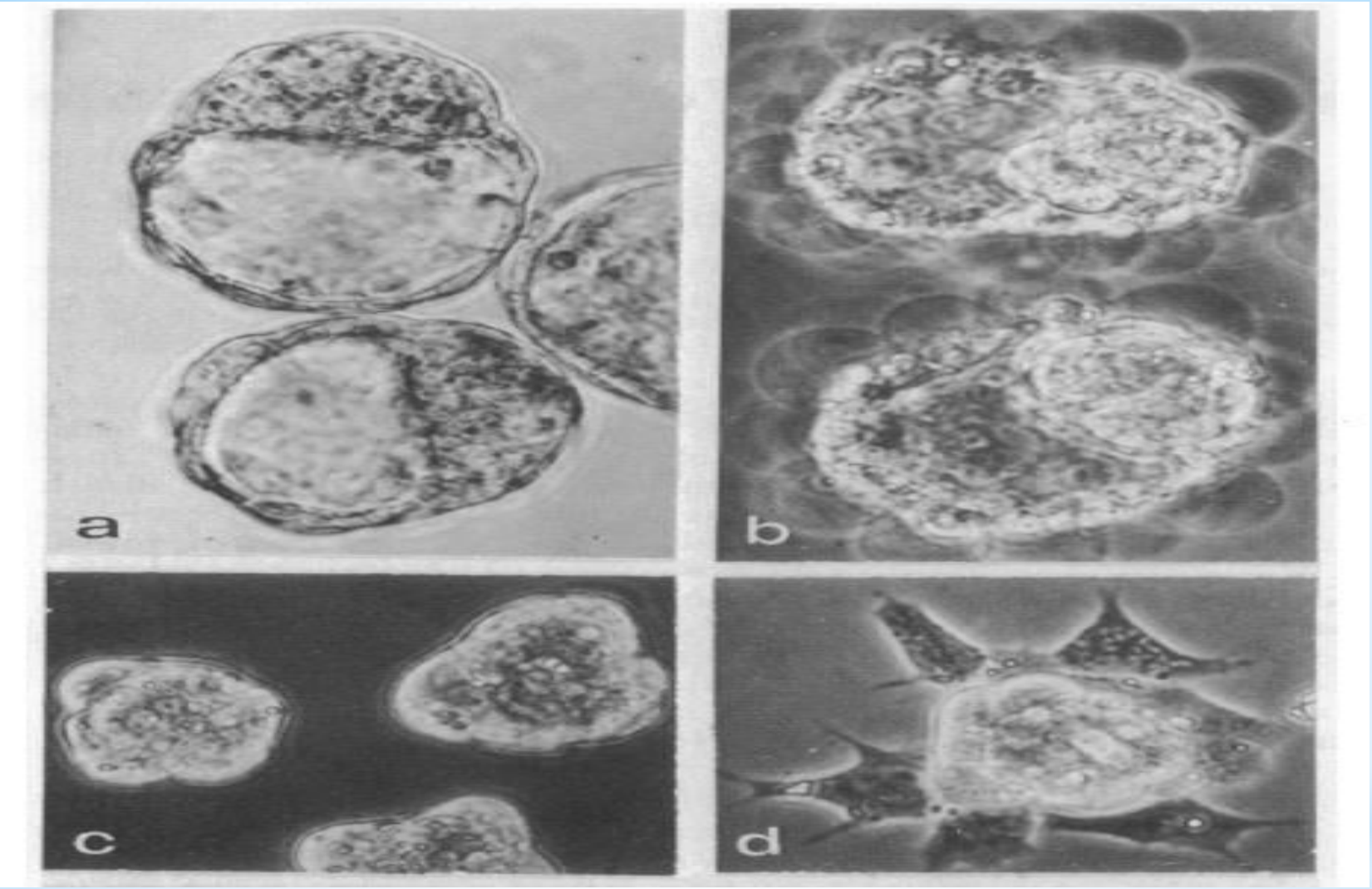
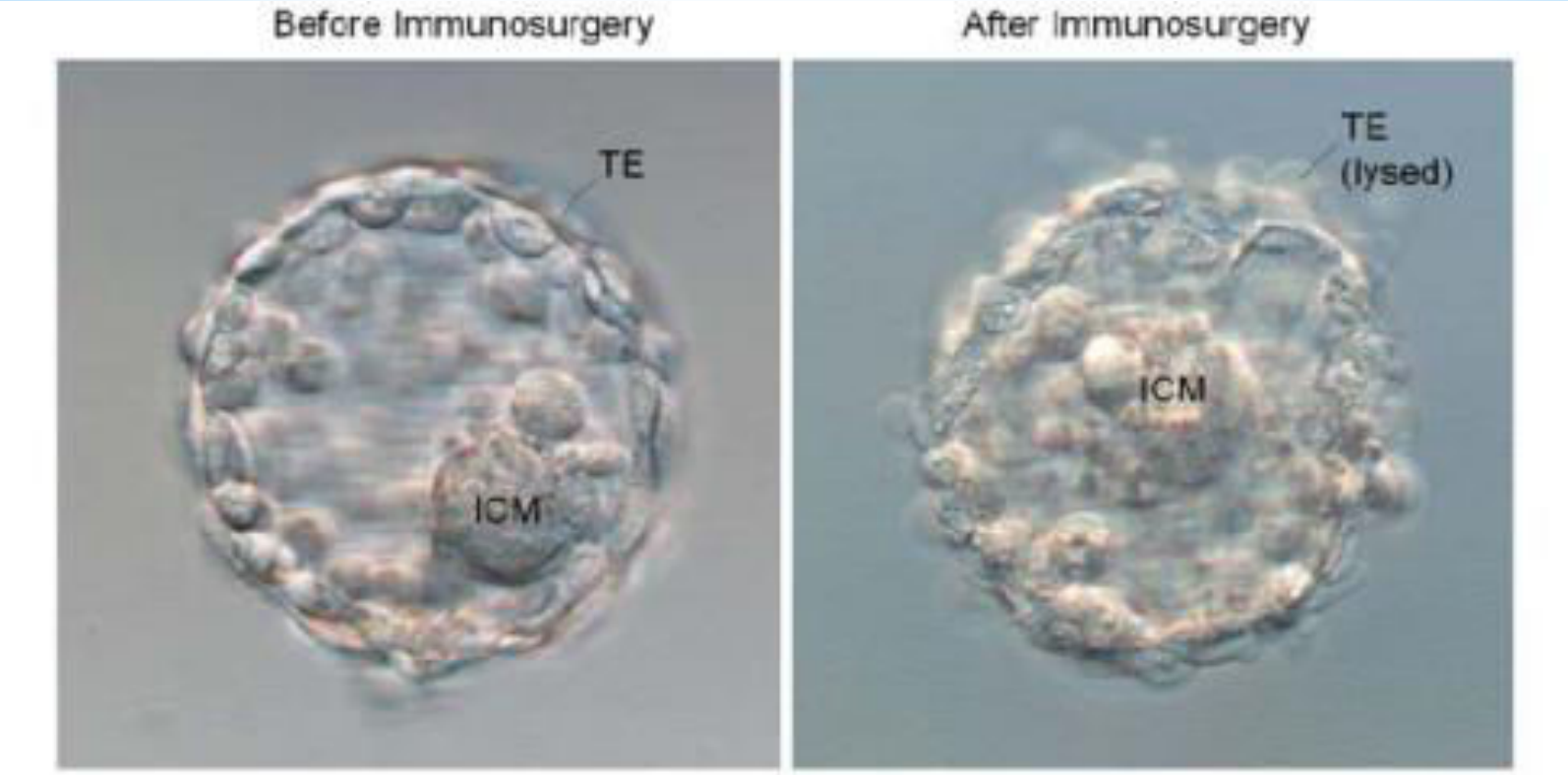


FIG. 2. Effect of two-step procedure on mouse blastocysts and their subsequent growth in culture. (a) Mouse blastocysts exposed to rabbit anti-mouse serum dilution 1:100 for 30 min; (b) blastocysts after exposure to antiserum and then to complement for 5 min—swelling of trophoblastic cells and demarcation of inner cell mass; (c) isolated inner cell masses cleared of dead trophoblastic cells; (d) inner cell mass after 24 hr in culture—attachment and outgrowth of cells containing vacuoles

Solter and Knowles, 1975


Before Immunosurgery
After Immunosurgery
TE
ICM
TE
(lysed)
ICM

Enzymatic isolation

- **Expanded Blastocysts**

  Pronase : for Zona digestion

  Trypsin : for T.E. digestion

  Trypsin inhibitors

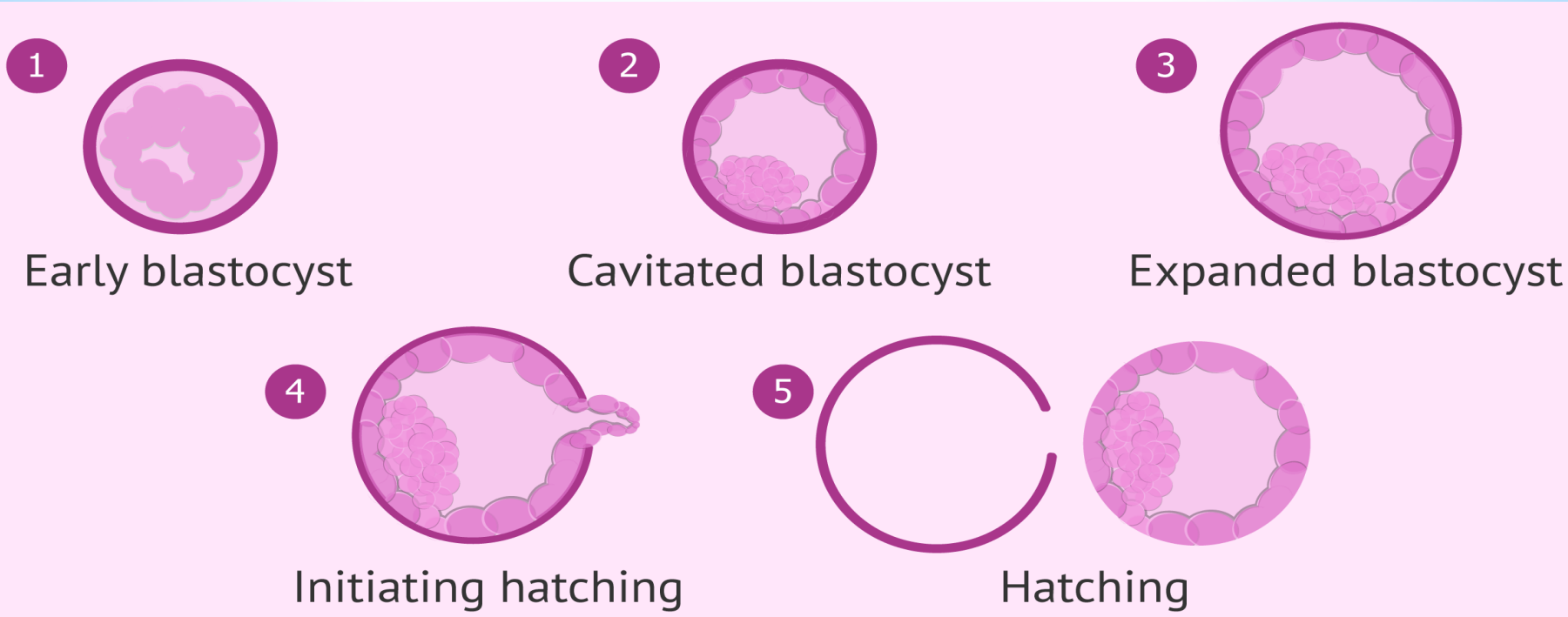
- **Hatched Blastocysts**

  Trypsin : for T.E. digestion

  Trypsin inhibitors

- **Enzymes like Collagenase, Trypsin, Dispase are commonly being used**

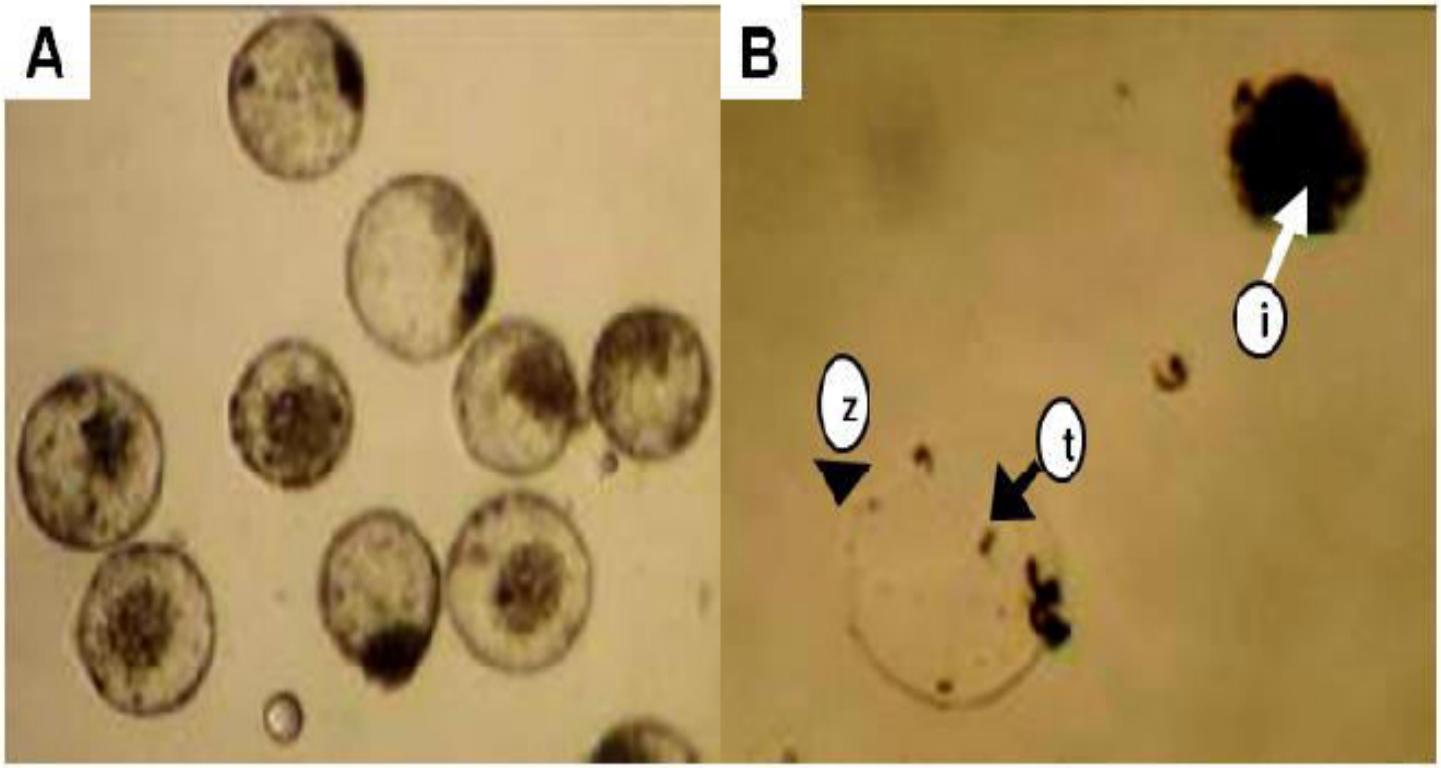
Grades of expansion of a blastocyst



Depending on its expansion, the blastocyst can be classified as early, cavitated, expanded, or hatching. The early blastocyst is the one that is initiating cavitation, i.e., the blastocele begins to form to give rise to the complete blastocyst structure. When it is cavitated, the blastocele has already formed, but it must continue to fill with liquid so that the blastocyst can expand. When the blastocyst is expanded, its size is larger and the zona pellucida is thinner, so hatching, i.e. the exit from the zona pellucida, can begin. Once it has hatched, implantation can take place.

Mechanical / Microdissectoin

- **ICM cells derived mechanical dissection and partial removal of trophoblast layer**
- **27G needle is used**
- **Isolation is performed under Zoom stereomicroscope**
- **Method suitable for hatched blastocyst (ICM visible)**
- **Laser assisted dissection to isolate ICM**

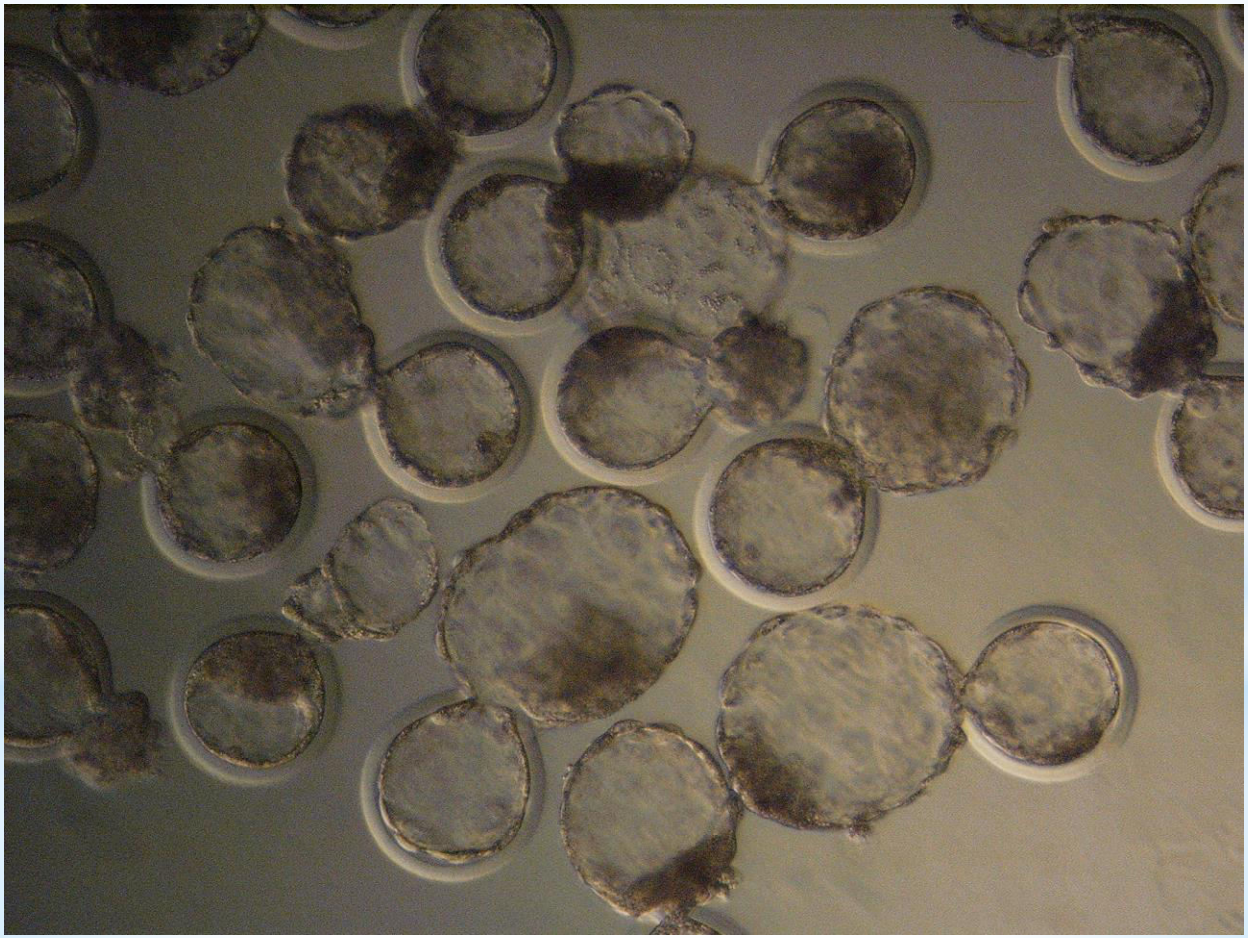


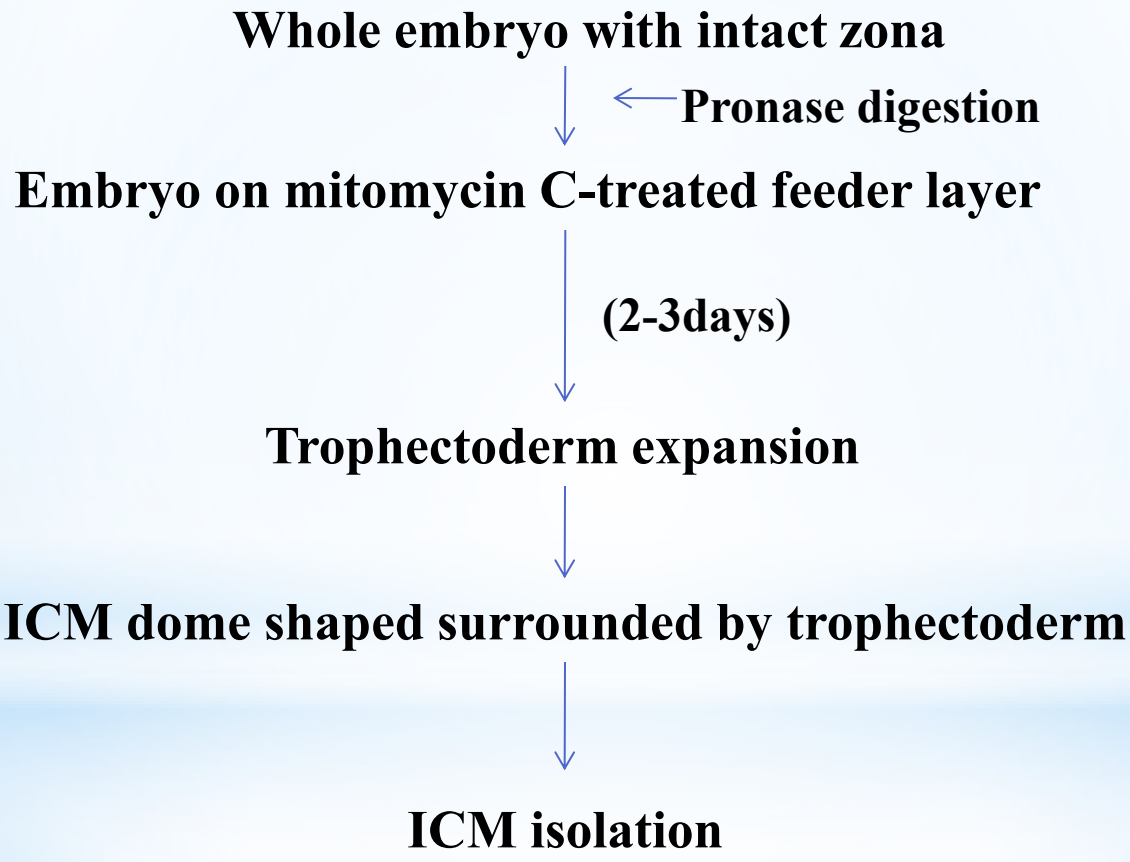
- Mechanically isolated bovine ICM. (A) Expanded and hatched blastocysts. (B) ICM (i) separated from the zona pellucida (**z**) and trophoblast cells (**t**).

Frietas *et al.*, 2011

Intact blastocyst culture

- Hatched Blastocyst seeded as such on feeder layer

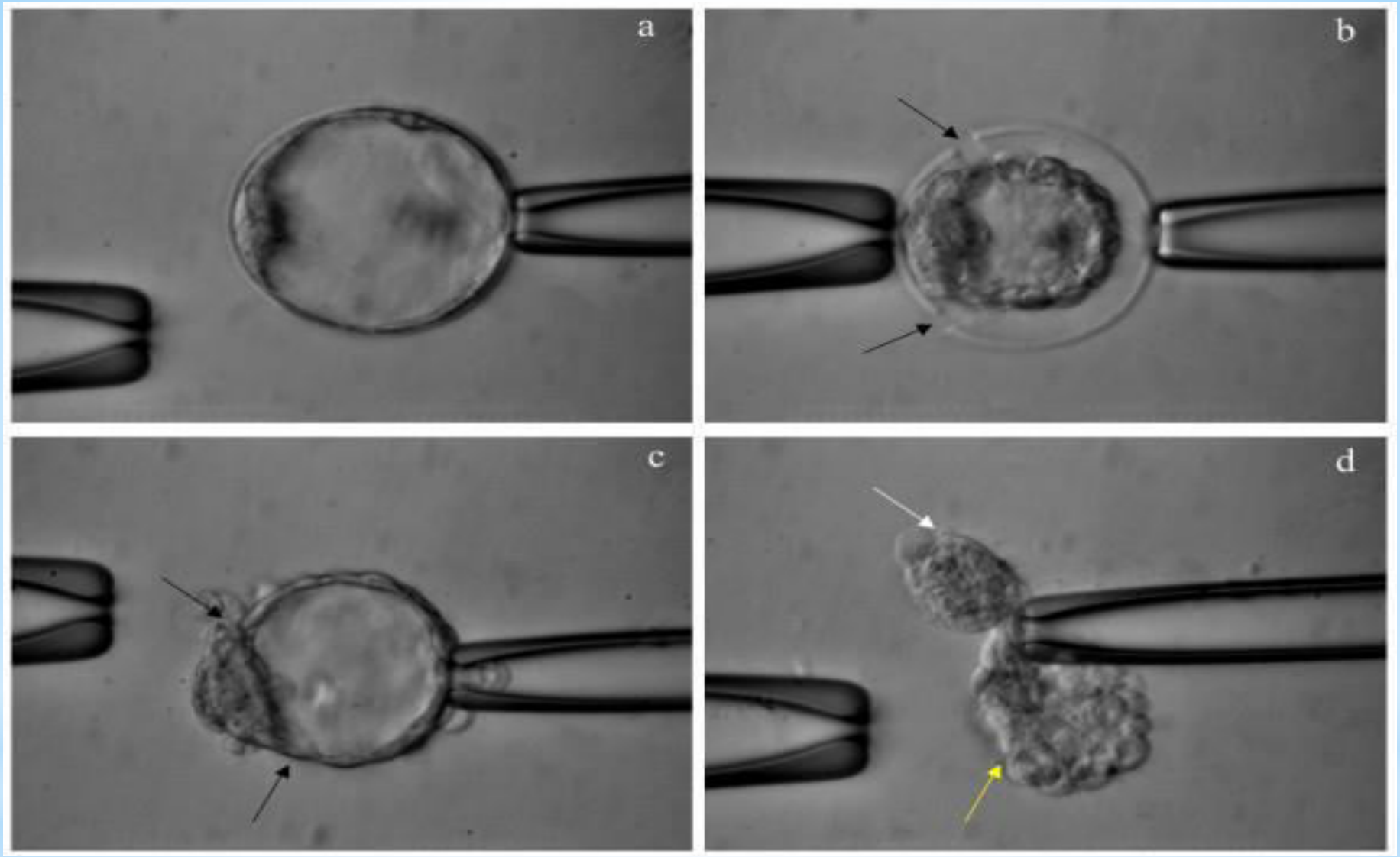



Whole embryo with intact zona
Pronase digestion
Embryo on mitomycin C-treated feeder layer
(2-3days)
Trophectoderm expansion
ICM dome shaped surrounded by trophectoderm
ICM isolation

Laser dissection

- Two holding pipettes holds blastocyst with ICM being positioned at 9o'clock.

- 10 infrared laser pulses are fired to split the blastocyst into two unequal portions-the smaller consisting of ICM, the larger consisting exclusively of trophoblast.



Laser dissection. Blastocyst secured by two holding pipettes with inner cell mass (ICM) being positioned at 9 o'clock before (a) and after (b) being sectioned by laser with (b) and without (c) zona pellucida. Arrows (b, c) indicate the resected area by laser energy. The smaller blastocyst fragment (white arrow) contains the ICM while the larger (yellow arrow) is exclusively trophoblast (d). Scale = 30 μm.

Tanaka N. *et al.*,2006

MAINTENANCE OF EMBRYONIC STEM CELLS

ES Cells require meticulous care , need to be maintained carefully and frozen, thawed and trypsinised with reasonable survival rates.

TWO TYPES OF CULTURE SYSTEMS

- **COCULTURE WITH FEEDER LAYER** (fetal muscle and fetal epithelial, adult epithelial, fallopian tube, marrow, fibroblast, and placental cells.)

- **FEEDER FREE CULTURE**

PREPARATION OF FEEDER LAYER

* **60-70% confluent fibroblast culture** is **either treated with mitomycin C** (10ug/ml) or **gamma irradiated** for mitotic inactivation
* **Mitomycin C covalently cross links the complementary strands of DNA** both *in vitro* and *in vivo* thus preventing DNA replication
* Secretes cytokines such as **Leukemia Inhibitory Factor (LIF)** preventing differentiation of stem cells.

LIF: Embryonic Stem Cells Maintaining Growth Factor

LIF (leukaemia inhibitory factor) is commonly used to maintain mouse embryonic stem cells in an undifferentiated state. Leukemia inhibitory factor (LIF) is **capable of maintaining embryonic stem (ES) cells in a pluripotent state through promoting self-renewal** or **suppressing stem cell differentiation**. It has become a standard protocol to use LIF to maintain murine embryonic stem cell pluripotency, whereas withdrawal of LIF allows embryonic stem cells to undergo cell differentiation. Upon withdrawal of both LIF and feeder cells, embryonic stem cells are able to, differentiate spontaneously into various cell types in three primitive layers.

Culture of bovine ICM on feeder layer

DMEM

supplemented with

10-20% **FCS**

.1mM **2-Mercaptoethanol**

hLIF – 10ng/ml

Streptomycin- 50ug/ml

Penicillin- 100 IU/ml

2mM L- **Glutamine**

Maintained at 37^0C, 5% CO_2 and 95% humidity

:**Delbecco's modified Eagle medium (DMEM)**

Fetal calf serum (FCS)

2-Mercaptoethanol also known as beta-mercaptoethanol is **a potent reducing agent used in cell culture media to prevent toxic levels of oxygen radicals**. 2-Mercaptoethanol is not stable in solution, so most protocols require daily supplementation.

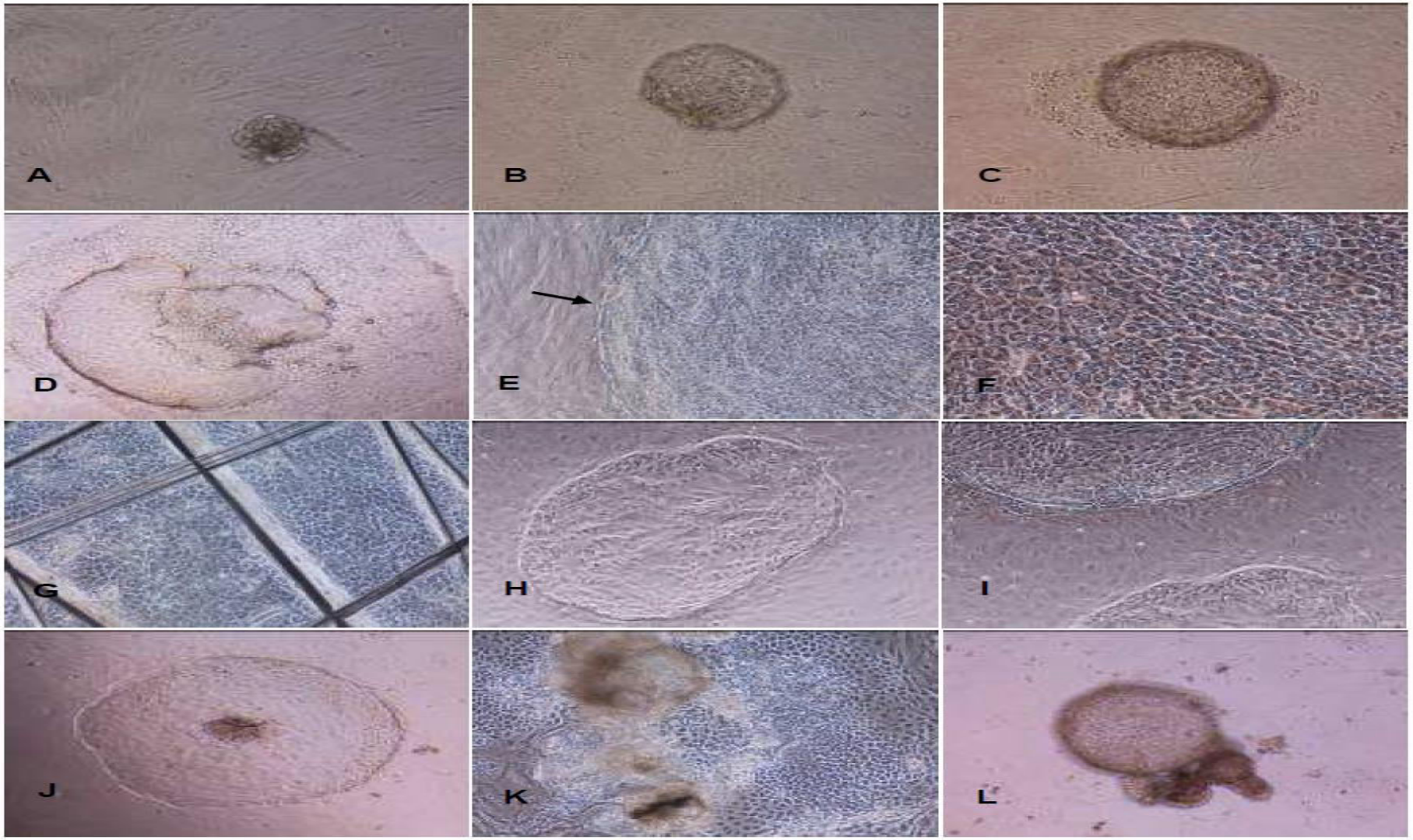


Figure 2 – Phase contrast micrographs of bovine ES-like cells and their differentiated derivatives. (A) A fresh isolated ICM over a MEF layer. (B) ICM expansion after attachment to MEF layer. (C) ICM outgrowth after 48 h isolation. Note that trophoblast cells were not seen. (D) Formation of a button in the middle of a large colony (primary colony). (E) Bovine ES-like cell colony after the first passage. Note the defined demarcation boundary of colony (arrow). (F) A higher magnification at the middle of the colony shown in (E). (G) Mechanical cut of bovine ES-like colony. (H-J) Flat colonies of bovine ES-like cells at different passages. (K) Bovine ES-like colony undergoing spontaneous differentiation. (L) Embryoid body (EB) from differentiated bovine ES-like cells. Magnification: x200 (A-C, F) and x100 (D-E, G-L).

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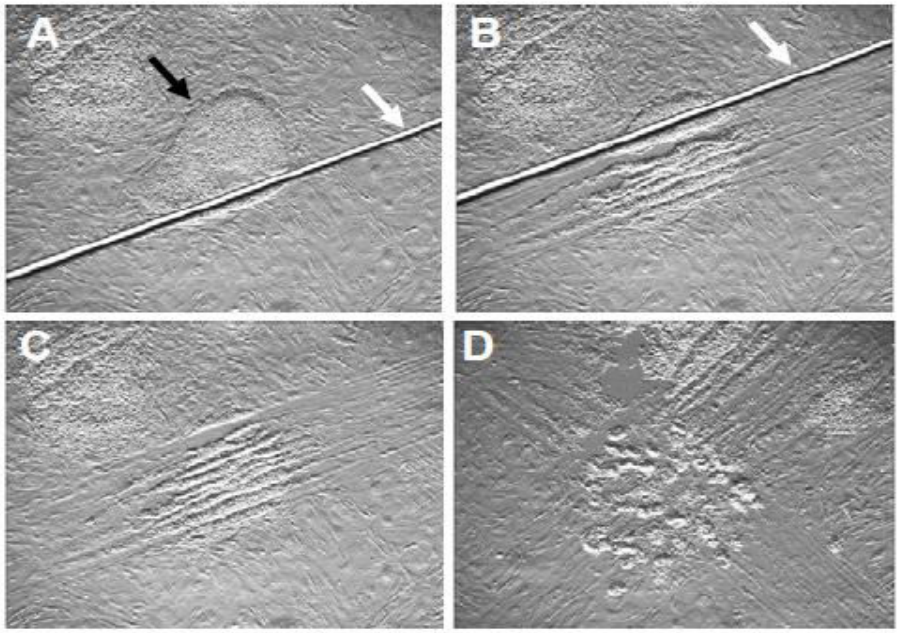


Figure 2. Microdissection passaging of hESC colonies. (A) An undifferentiated hESC colony growing on high density MEFs (black arrow). (A,B,C) A drawn out glass Pasteur pipette (white arrow), is pressed into the colony, to make a series of cuts. (D) An series of perpendicular cuts divides the colony into a grid of largely uniform pieces. The pieces are lifted from the plate with the glass needle. All the undifferentiated colonies in the dish are manipulated this way, and the pieces transferred to a new dish.

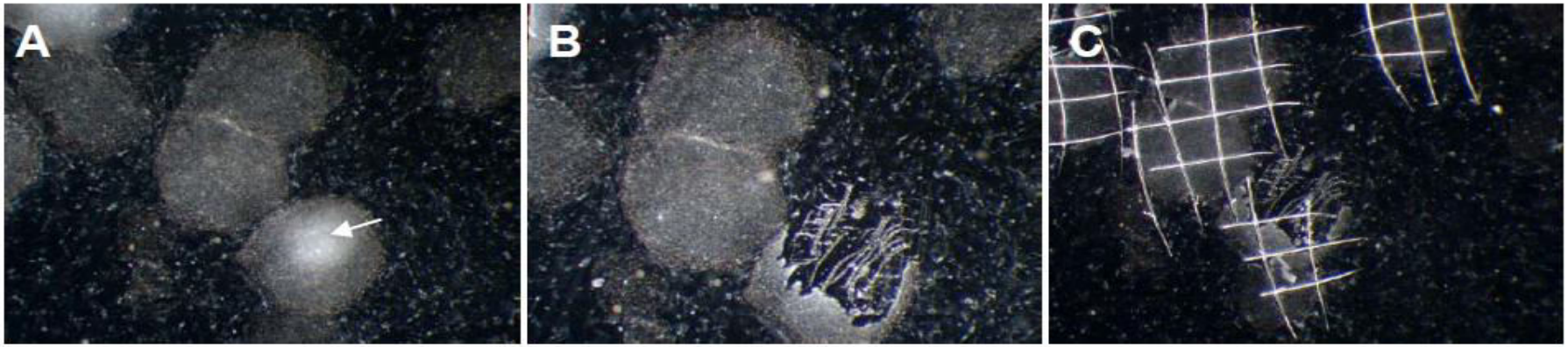


Figure 3. Microdissection passaging of hESC colonies on low density MEFs. (A) A hESC colonies viewed under a dissection microscope. Differentiated colony areas appear white (white arrow), while undifferentiated colonies are opaque. (B) Differentiated areas are excised and discarded. (C) The remaining undifferentiated colonies are scored with a needle (white lines), before lifting off and passaging.

Feeder free culture system (Defined medium)

* **Matrigel** or **Laminin** coated plastic plates
* MEF conditioned medium
* KOSR

Fibronectin matrix

Supplements-

In hESC:

KOSR

Transforming growth factor-b (**TGF-b1**),

Fibroblast growth factor (**bFGF**),

In mESC:

Leukemia inhibitory factor **(LIF)**

Matrigel is **commonly used as a 3D matrix for cell culture and tissue engineering, providing a supportive environment for cell growth and differentiation**. MatriGel is also utilized as a drug delivery system, enabling controlled release of therapeutic agents.

Matrigel is **used to pre-coat cell culture dishes and provide an attachment surface for feeder-free human iPS cells to replicate on**. We use Matrigel from Corning (product number) that is provided in a glass bottle as a concentrated liquid (5ml) with lot-specific dilution information.

What is the purpose of Matrigel?

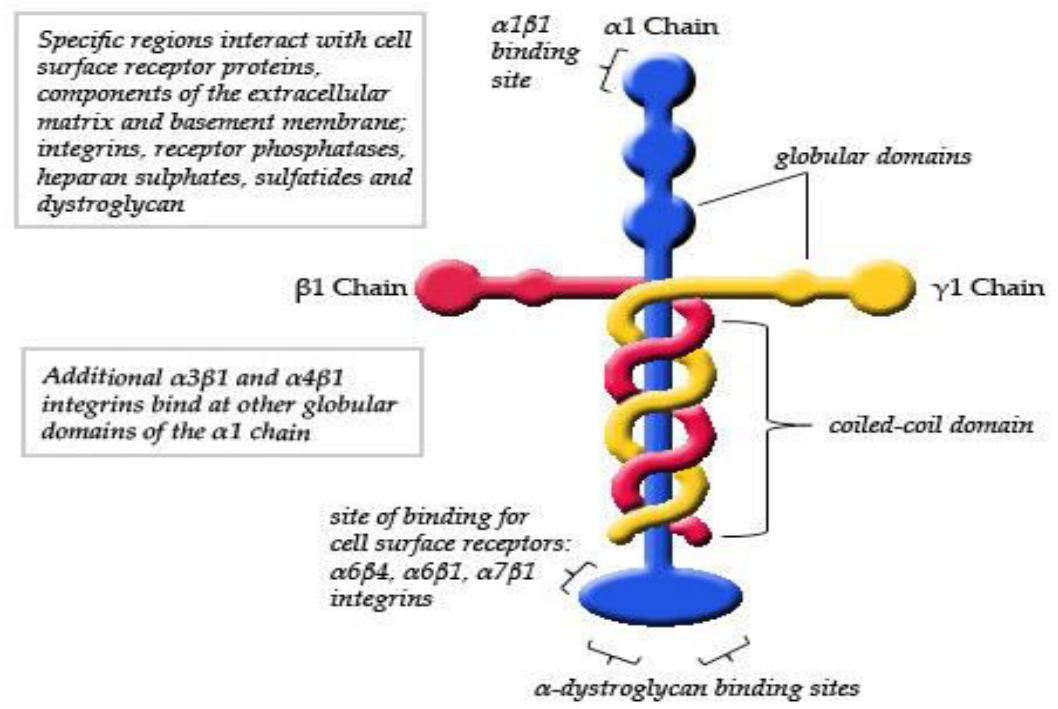
Matrigel has been used extensively as **a surface coating for 2D cultures of human PSCs as a replacement for stromal feeder layers**. More recently, Matrigel has been used as an essential **3D matrix to permit the self-organization of stem cells into organoid structures**.

Matrigel is **an extract obtained from the Engelbreth-Holm-Swarm mouse sarcoma** and composed chiefly of **collagen IV**, **laminin**, **entactin**, and **heparin sulfate proteoglycans**.

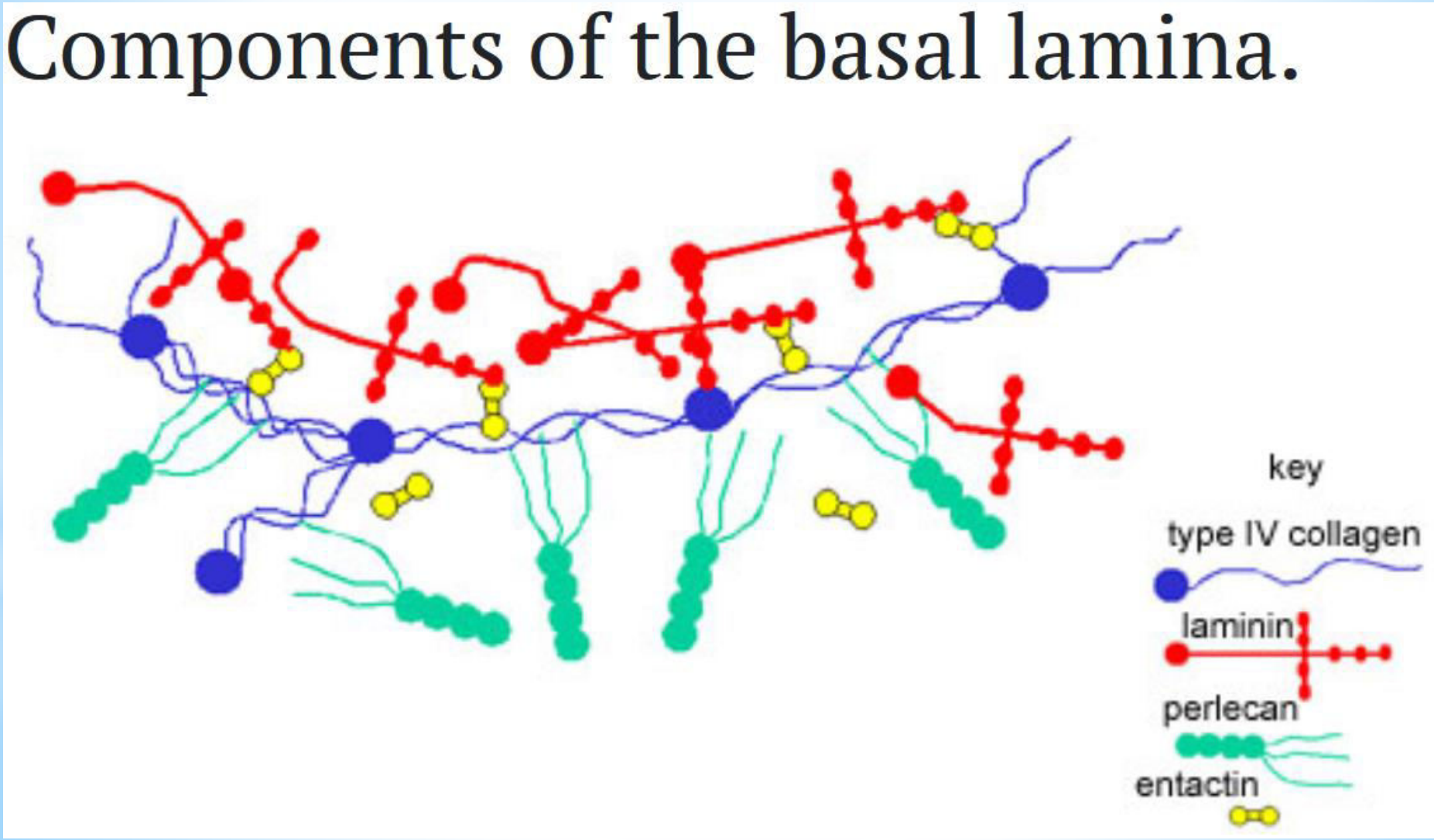
What is the composition of Matrigel?

Corning Matrigel matrix is a reconstituted basement membrane preparation that is extracted from the **Engelbreth-Holm-Swarm (EHS) mouse sarcoma**, a tumor rich in extracellular matrix proteins. This material, once isolated, is approximately **60% laminin, 30% collagen IV, and 8% entactin**.

Laminins are **a family of glycoproteins that consist of one heavy α chain and two light β and γ chains** (Timple, 1989). The laminin molecule is a major component of the basement membrane and plays important roles in cell differentiation, adhesion, and migration (Timple, 1989).



Entactin has the ability to **form a link between laminin and type IV collagen**, the two most abundant basement membrane molecules. As a bridge molecule, it plays an important role in maintaining the integrity of the base- ment membrane.



What is the function of KnockOut serum replacement?

Knockout serum replacement (KOSR) is a nutrient supplement commonly used to **replace serum for culturing stem cells**. We show here that KOSR has pro-survival activity in chronic myelogenous leukemia (CML) cells transformed by the BCR-ABL oncogene.

What are the ingredients in Kosr?

In essence, KOSR is **a mixture of small organic molecules (amino acids, vitamins and antioxidants), trace elements and three proteins, namely insulin, transferrin and lipid-rich albumin**. Of these, the lipids associated to albumin are the only non-chemically-defined component of KOSR.

What is the main function of fibronectin?

Fibronectin is an adhesive glycoprotein that is primarily involved in **cell–adhesive interactions**. Intracellular signaling induced by cell adhesion on fibronectin plays a critical role in **cytoskeletal organization**, **cell cycle progression**, **growth** and **cell survival and differentiation**.