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Immunocytochemistry in Early Mammalian Embryos

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1. Introduction

The preimplantation period of mammalian development hosts very important cellular and molecular events. This period starts with the fertilization of oocyte by sperm, a process that reprograms the highly differentiated nuclei of these germ cells, and leads to the generation of a totipotent one-cell embryo. Then, the embryo performs cleavage divisions with short cell cycles to quickly increase its cell number. During this period, the genome of the preimplantation embryo manifests profound changes in nuclear and chromatin organization, histone modifications, and transcriptional activity. These genome alterations are also coupled to cell signaling pathways and their regulatory effects. The final product of the preimplantation development is a multi-cellular blastocyst containing three types of cells, epiblasts, hypoblasts, and trophoblast cells [1].

To study and understand the biology of preimplantation embryos, different techniques have been used. The paucity of cells and the difficulties associated with the preparation and production of preimplantation embryos have been the main limiting factors for the application of a wide range of experimental techniques. Thus, what is known about early embryos today is mainly the results of the use of a few experimental techniques and their adapted modifications. These include DNA and RNA amplification techniques, transcript labeling, *in situ* hybridization of DNA and RNA, gene manipulation studies, and light, electron, and immunofluorescence microscopy techniques.

The application of each technique has revealed a specific aspect of preimplantation developmental biology. Table 1 summarizes and compares the contributions of different experimental techniques applied on preimplantation mammalian embryos. In the rest of this chapter, I will focus on the immunocytochemical staining of embryos and its different applications in preimplantation development.

2. Contribution of immunocytochemistry to understanding the biology of preimplantation mammalian embryos

Application of immunocytochemistry (ICC) on preimplantation embryos has provided invaluable information on different aspects of preimplantation development. I will briefly

Technique	Knowledge contribution	Example references
Conventional and quantitative RT-PCR	Evaluation of the transcription of individual genes	[2-8]
Gene expression profiling (microarray)	Large-scale evaluation of the expression of genes	[9-14]
Electron microscopy techniques	Studying the ultrastructural organization of the embryonic cells	[15-19]
Labeling of nascent transcripts	Quantification of transcriptional activity	[20-24]
In situ hybridization of DNA and RNA	Intracellular localization of chromosomes and transcripts	[25-28]
Gene knockout and knock down techniques	Studying the function of individual genes	[29-38]
Immunocytochemistry	Intracellular localization of proteins Quantitative evaluation of the expression of proteins Identification of protein modifications Evaluation of the activity of certain signaling pathways	[20, 31, 39-45]

Table 1. The major Experimental techniques applied to study the preimplantation embryos.

review the applications of ICC for localization of proteins, for studying the modifications of chromatin and alteration of chromatin organization, and for analyzing cell signaling pathways in preimplantation embryos.

2.1 Cellular and intra-cellular localization of proteins

During preimplantation development, it is very important to identify whether a given protein is expressed, where in the cell it is localized, in which blastomeres it is expressed, and when its expression is eliminated. All of this information relate to the function of protein during preimplantation development. Immunocytochemistry has been an indispensable technique to reveal this information. Application of an alternative Western blotting will not provide any information on the intracellular localization of the protein or the types of expressing cells.

Looking at more than two decades of research on Oct4 clearly shows that what we know on the role of this transcription factor in pluripotency, has all started from this immunocytochemical observation that this protein is differentially expressed in the mouse preimplantation embryonic cells [46]. While it had been previously revealed that it has a strong transcriptional activator effect in the inner cell mass of the preimplantation embryo [47] and it is transcribed in these cells [48], it was its protein localization (using specific antibodies and ICC procedure) that convincingly illustrated its relationship to stemness and pluripotency. A number of later functional studies also used ICC to reveal the function of Oct4 during preimplantation development and pluripotency [49, 50]. The same route of discovery has been traveled for other stemness genes [51].

Using immunocytochemistry and confocal microscopy we have been able to reveal the subcellular distribution and to analyze the relative amount of ten isozymes of PKC (alpha, betaI, betaII, gamma, delta, epsilon, eta, theta, zeta, iota/lambda) and a PKC-anchoring protein, receptor for activated C-kinase 1 (RACK1), between the two-cell and blastocyst stages of mouse preimplantation development [39]. In a functional study, we used the same principle to analyze the relative amount of each PKC isozyme within each blastomere and relate this to the transcriptional activity of the 4-cell mouse embryo [20]. Thus for a given protein in the preimplantation embryo, ICC technique can be applied to study its differential expression between embryonic blastomeres, to identify its intracellular localization within individual blastomeres, and also to semi-quantitate its expression. Recently, using fluorescently-labeled specific antigen binding fragments (Fabs), it has been shown that it is possible to monitor the distribution and global level of endogenous histone modifications in living blastomeres without disturbing cell growth and embryo development [52].

2.2 Identification of histone modifications and the study of nuclear organization

The last two decades has witnessed a considerable number of research efforts using ICC to identify a variety of post-translational modifications on histones and to analyze the expression of chromatin-remodeling factors in preimplantation embryos (Table 2). Immunocytochemical detection and localization of nuclear subdomains (Figure 1), histone modifications, enzymes responsible for these modifications, different histone variants, distinct chromatin remodeling factors, and the status of transcription in preimplantation stages of development (Figure 2), has provided ample evidence and knowledge on the biology of chromatin during preimplantation development (Table 2).

In a very close subject, ICC procedure has also been applied to investigate the organization of chromatin, the architecture of nucleus, and the formation of sub-nuclear compartments by ultra-structural studies in preimplantation embryos. In fact, the correlative fluorescence and electron microscopy technique has allowed the ultra-structural identification of nuclear entities which are identified and tagged by immunocytochemistry [15, 53, 54] (Figure 3). As it has been shown in the figure, immunocytochemical detection of a chromocenter domain immuno-stained with CREST antibody is indispensable for finding and imaging it under the electron microscope. The same principle has been used to identify a sub-nuclear compartment immunocytochemically, and to study its ultra-structure, e.g. localizing fibrillarin by ICC to identify nucleolus in the nucleus of preimplantation embryos for ultra-structural analysis [19, 55-57].

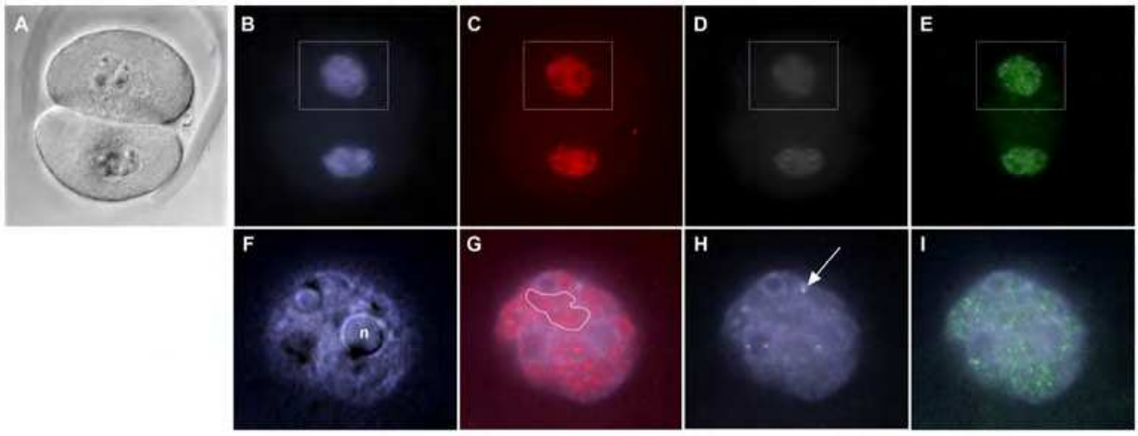

Fig. 1. Immunocytochemistry and confocal imaging of a two-cell mouse embryo to evaluate the function of nucleus. Top row contains confocal images from an optical slice of a two-cell mouse embryo which has been immunolabeled and stained with different antibodies and imaged in different channels. The bottom row contains the merged images of the top nucleus in different channels. A two-cell embryo (A; DIC image) contains two nuclei that are not very chromatin-condensed by DAPI staining (B). Nucleoli (n) in the magnified nucleus in F (the merged image of A and B) show very thin rim of fairly condensed chromatin. Immunolabeling of RNA polymerase II (phosphorylated at serine 5 of its CTD) shows a hyperactive transcription (C). A highly transcribed region of nucleus has been marked in G (the merged image of B and C). Immunolabeling with CREST antibody reveals centromeres (D), which are mainly located at the edge of nucleoli in H (the merged image of B and D). White arrow in H, shows a CREST-labeled spot. Immunolabeling with the antibody against acetylated lysine of H3 histone reveals regions of "open" chromatin (E) which are distributed throughout the nucleus (I, the merged image of B and E).

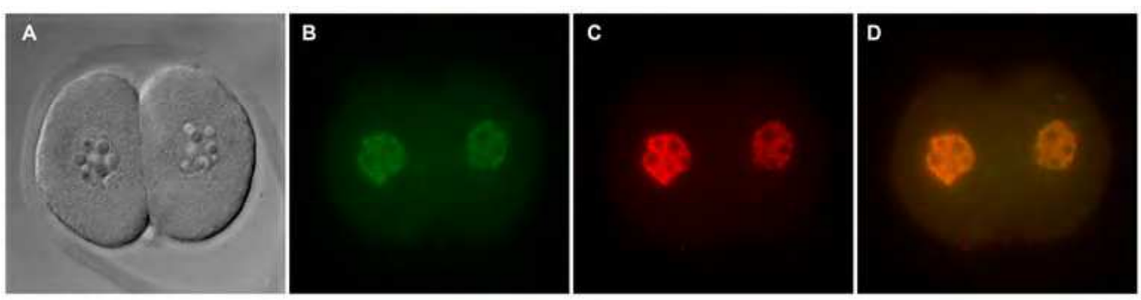

Fig. 2. Immunocytochemical localization of hyperactive transcription domains in a two-cell stage mouse embryo. A) DIC image; B) Immunolabeling with the antibody against the acetylated lysine of histone H3; C) Immunolabeling with the antibody against RNA polymerase II (phosphorylated at serine 5 of its CTD); D) A merged image of B and C. The yellow color in D represents nuclear domains which contain acetylated H3K9 and RNA pol II, indicating that transcription is occurring in chromatin domains with a relaxed state, where a large number of acetylated histone H3K9 moieties are present.

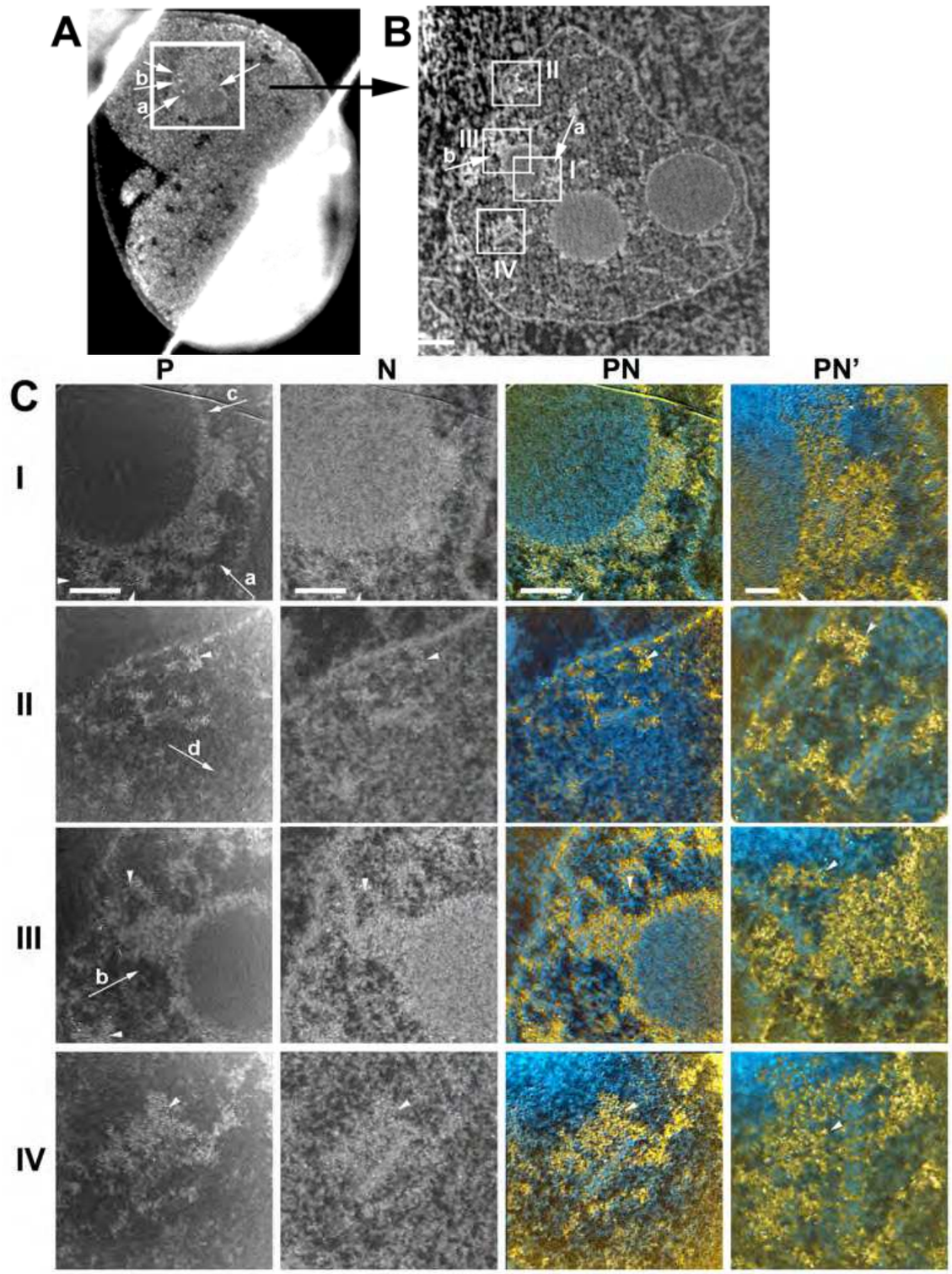

Fig. 3. Chromatin organization in the two-cell stage preimplantation mouse embryo. **A)** Fluorescence image of a physical section spanning through the nucleus (shown by the white

box) of a two-cell stage embryo. White arrows point to the centromeres which are immunostained with CREST antibody. **B)** The rectangular region in panel A has been imaged by low magnification electron spectroscopic imaging (ESI; 155 keV phosphorus-enriched)[15, 53, 54, 58, 59]. Three different-sized nucleoli with very homogenous mass are noticeable in this nucleus. Arrows 'a' and 'b' point to the centromeres designated similarly in panel A. The scale bar is 2 nm. **C)** Different regions of the nucleus in panel B have been imaged with higher magnification ESI. Columns P, N, PN, and PN' denote images of phosphorus map, nitrogen map, overlay of phosphorus and nitrogen maps, and higher magnification overlays of phosphorus and nitrogen maps, respectively. The segmentation of signals in PN and PN' permits the visualization of chromatin fibers as yellow, while non-chromosomal proteins due to their relatively low N:P ratio content are in blue color. White arrowheads point to the representative gold-tagged histone H3 (methylated at lysine 9) molecules which are accumulated at different areas of the nucleus. Scale bars for columns P, N, and PN are 500 nm, and scale bar for column PN' is 200 nm. (C-I) Nucleoli comprise a homogeneous structure with scarce amounts of ribonucleoprotein (weak signal in P map), but large amounts of protein (strong signal in N map). A more condensed patch of chromatin at the edge of nucleolus (arrow a) is highly positive for K9-methylated H3, while a very thin layer of chromatin at the edge of nucleolus (arrow c) does not show accumulation of this signal. The area shown by 'arrow a' which is designated similarly in panels A and B corresponds to a chromocenter. **(C-II)** A very thin layer of condensed chromatin (as 30nm fibers) at the nuclear envelope which in some parts is positive for K9-methylated H3 blends in with the open lattice of 10nm chromatin fibers (shown by arrow d). The open lattice is filled with large amounts of non-chromosomal proteins shown as blue in PN image. The relation of chromatin and non-chromosomal proteins is better visualised in the higher resolution/magnification image of PN'. **(C-III)** Patches of condensed chromatin at the edge of nucleolus and in the vicinity of nucleolus (white arrowheads) are positive for K9-methylated H3, but only the area at the edge of nucleolus (shown by arrow b) corresponds to the chromocenter 'b' in panels A and B. **(C-IV)** Non-centromeric condensed chromatin (as 30 nm fibers and positive for K9-methylated H3) is surrounded by dispersed network of 10nm fibers.

2.3 Evaluation of the activity of certain signaling pathways

Immunocytochemsitry has also been used to discover the presence of many components of signaling pathways including Wnt, hedgehog, receptor tyrosine kinase, and PKC in preimplantation embryos. These studies based on imaging and localization of specific proteins has clearly established a framework for future functional studies. In Table 3 some of these studies have been summarized.

3. Immunocytochemistry of oocytes and preimplantation mammalian embryos

3.1 Harvesting oocytes and preimplantation embryos

Depending to the species, oocytes can be acquired and preimplantation embryos can be produced in different ways. In mouse, it is very easy to harvest from oviduct and uterus, the oocytes and embryos grown in vivo to certain stages of preimplantation development. It is also possible to harvest embryos at early cleavage stages and grow them in culture medium

Findings	Implication	Example references
Lack of the constitutive heterochromatin markers histone H4 trimethyl Lys20 (H4K20me3) and chromobox homolog 5 (HP1α); the presence of heterochromatin markers, H3K9me3, 5-methyl cytosine (5MC), HP1β, H3K27me3, H4K20me1 and H4K20me2	Heterochromatin is in an immature state in mouse preimplantation embryos	[60]
Presence of the acetylated forms of H3K9 and H3K27	H3K27 acetylation is important for normal embryonic development	[52]
Relatively higher expression in oocytes and early cleavage stage embryos of methionine adenosyltransferase 1A protein up to the 8-cell stage compared with the morulae and blastocyst stages	nutrient-sensitive epigenetic regulation and perturbation may be performed through specific enzymes at the earliest stages of preimplantation development	[61]
Embryos at 2-, 4-, and 8-cell stages lack macroH2A except in residual polar bodies. MacroH2A protein expression reappears in embryos after the 8-cell stage and persists in morulae and blastocysts, where nuclear macroH2A is present in both the trophectodermal and inner cell mass cells.	Normal embryos execute three to four mitotic divisions in the absence of macroH2A prior to the onset of embryonic macroH2A expression. Embryos made by somatic nuclear transfer utilize the same chromatin remodeling mechanisms.	[62, 63]
HDAC1 is expressed in preimplantation embryos , where its expression inversely correlates with changes in the acetylation state of histone H4K5 during preimplantation development	HDAC1 is involved in the formation of a chromatin-mediated transcriptionally repressive state that initiates in the late two-cell embryo	[31]
ICC of late zygotes shows that constitutive heterochromatin is only maternally labeled by H3K9me3 and HP1β	In early embryos, Suv39h-mediated H3K9me3 constitutes the dominant maternal transgenerational signal for pericentric heterochromatin formation	[34]
After fertilization, level of H3K79me2 and H3K79me3 modifications rapidly decrease, and the hypomethylated state is maintained at the interphase (before the blastocyst stage), except for a transient increase in H3K79me2 at mitosis (M phase). H3K79me3 is not detected throughout preimplantation, even at M phase	Elimination of H3K79 methylation after fertilization is involved in genomic reprogramming	[64]
p150CAF-1 is expressed in preimplantation embryos and loss of p150CAF-1 function leads to early developmental arrest and alteration of heterochromatin organization	Chromatin assembly machinery is involved in controlling the spatial organization and epigenetic marking of the genome in early embryos	[42]

Table 2. Immunocytochemical identification and analysis of some histone modifications and chromatin remodeling factors in preimplantation embryos.

Findings	Pathway*	Example references
Expression of protein kinase C isoforms in each stage of preimplantation development	Activation of PKC through G-protein coupled receptors	[39, 65]
Expression of Hh receptor PTCH1 and co-receptor SMO	Signaling events mediated by the Hedgehog family	[66]
Expression of β-catenin	Wnt signaling network	[67-69]
Presence of Aurora C in cleavage-stage embryos	Signaling by Aurora kinases	[70]
Expression of proteins in MAPK pathway	p38 MAPK signaling pathway	[71]
IRS-1 is expressed in all cell lineages of the peri-implantation mouse embryo and mediates some effects of insulin and IGFs at this stage.	Insulin pathway	[72]
Expression and localization of beta 1, beta 5 and alpha 6 integrins and ZO-1 and E-cadherin proteins	E-cadherin signaling pathway & integrin family cell surface interactions	[73, 74]
Strong expression of c-MYC signal in the nucleus of growing and fully grown oocytes as well as in preimplantation embryos before the morulae stage	C-MYC pathway	[75]
The p 85 and p110 subunits of PI3K and Akt are expressed from the 1-cell through the blastocyst stage of murine preimplantation embryo development	The PI3K/Akt pathway	[76]

*Name of pathways have been adapted from NCI-Nature Pathway Interaction Database [77]

Table 3. Components of signaling pathways immunocytochemically identified in preimplantation embryos.

(in vitro culture; IVC). In addition, the early embryos could be produced by in vitro fertilization (IVF) of oocytes, and subsequently cultured in vitro. The most practical method to acquire bovine embryos is through IVF followed by IVC.

Superovulation of female mouse: In mouse, whether we need oocytes, in vivo grown embryos, or in vitro fertilized and cultured embryos, the female mice required to be

superovulated. In response to a hormonal regimen, 3 weeks-old female mice produce the highest number of oocytes (metaphase II stage) and embryos. This is believed to be related to the lack of reproductive cycles and an inactive state of hypothalamic-hypophysial-gonadal axis at this age. The acquired number of harvested oocytes and embryos after superovulation is also largely affected by the strain and maintenance (nutritional and light-dark cycle) conditions.

To induce superovulation of female mice, the following steps need to be taken.

1. Mice should be kept in a 12 hour light-dark cycle in a properly ventilated room with a temperature of 22-26°C.
2. Administration of hormones is performed by intra-peritoneal injection of female mice at 3-weeks age. If the mice are bred in the same facility, then the first injection time would be two days after weaning from mother. However, if mice will be transferred to the facility from another location, then the first injection time would be after a two-day acclimatization period.
3. Human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG), which are in the lyophilized powder form, should be dissolved in sterile saline solution (0.9% NaCl) under a laminar hood. The final concentration is 5 IU per 0.1 ml. Once all the powder in each vial has been dissolved, 0.5 ml of each solution should be drawn into individual insulin syringes and immediately placed in -80°C freezer.
4. Each 3 weeks-old female mouse is injected intra-peritoneally at 14pm on day -3 with 0.1 ml of PMSG (5 IU). The syringe containing the hormone should be removed from freezer and brought to the ambient temperature 15 minutes before injection.
5. On day -1 at 12pm (46 hours after PMSG injection), each injected mouse will be injected again with 0.1 ml of hCG (5 IU) intra-peritoneally. If harvesting of embryos is intended, each female mouse after injection should be placed in the cage of individual males (Note 1) for overnight mating. However if oocyte recovery is anticipated, the females are returned to their own cage after second injection.

Harvesting oocytes: In the morning of day 0, oocytes can be recovered from oviducts of injected females. Oviducts are flushed with M2 medium (Sigma-Aldrich, St Louis, MO, USA) as previously described [78]. It should be noted that the oocytes at this stage are surrounded by layers of granulosa cells. Thus, to perform ICC and properly localize and image specific proteins in oocytes, the granulosa cells need to be digested away. Otherwise, it will not be possible to properly image oocyte itself, especially when an epi-fluorescence microscope is used for imaging.

Harvesting embryos: To harvest embryos, the injected female is placed in male's cage for overnight mating. Presence of a copulation (vaginal) plug the next morning (on day 0), would be an indication for mating. Embryos at different stages of preimplantation development can be harvested at different time points. Table 4 represents approximate time points for the recovery of embryos at different stages of mouse development.

3.2 Immunocytochemistry

Oocytes or embryos do not attach to the slides or coverslips. Thus, the ICC procedure on harvested oocytes or embryos is somewhat different from the ICC procedure performed on cells grown on coverslips or cells attached to slides. During the procedure, oocytes and

Stage	Day	Time
One-cell stage	0 (The day after hCG injection and mating*)	10-12 am
Two-cell stage (most likely at G2 phase of cell cycle)[79]	1	9 am (45 hours after hcG injection) (33 hours post coitum*)
Four-cell stage (G1 or S phase of the cell cycle)[79]	1	4 pm (52 hours after hcG injection) (40 hours post coitum)
Eight-sixteen cell stage	2	9 am (69 hours after hcG injection) (57 hours post coitum)
Morulae stage	2	4 pm (76 hours after hcG injection) (64 hours post coitum)
Early blastocyst	3	9 am (93 hours after hcG injection) (81 hours post coitum)

* When male and females are placed in a cage for mating in an evening, the 12:00 midnight is arbitrarily chosen as the time of mating.

Table 4. Approximate time points for the recovery of embryos at different stages of mouse preimplantation development.

embryos should be manually transferred between different media containing fixative, permeabilizing agent, or antibodies. Use of depression slides as container and a stereomicroscope would facilitate the procedure. Pipettors (e.g. 20 μl) or mouth-controlled pipet devices [78] are used for the transfer, while embryos are watched under the stereomicroscope.

The following procedure is a prototype to perform ICC (using fluorescent secondary antibodies) on oocytes and embryos. For simplicity, only embryos (not oocytes) are referred to in the procedure.

1. **Washing:** Wash embryos in 200 μl of PBS twice. This will involve the quick transfer of the harvested embryos into the depression slides containing PBS. Under the stereomicroscope, the embryos could be counted and screened for fragmented or abnormal morphology.

2. **Fixation:** Transfer embryos into 200 µl of 4% paraformaldehyde in PBS and incubate at room temperature for 20 minutes. After fixation, the embryos are washed in PBS three times (of 5 minutes each) at room temperature. At this step, the embryos can be stored in PBS at 4°C overnight.
3. **Permeabilization** (Note 2): Transfer fixed embryos into 200 µl of 0.5% Triton X 100 in PBS and incubate for 5 minutes at room temperature. Wash the permeabilized embryos in PBS three times (of 5 minutes each) at room temperature.
4. **Incubation in primary antibody:** Transfer embryos into 200 µl of primary antibody (diluted in PBS). Incubate in a humid chamber for 2 hours at room temperature or overnight at 4°C. Wash the embryos in PBS three times (of 5 minutes each) at room temperature (Note 3).
5. **Incubation in secondary antibody:** Transfer embryos into 200 µl of secondary antibody (diluted in PBS). Incubate in a humid chamber for 1 hour at room temperature or overnight at 4°C. Wash the embryos in PBS three times (of 5 minutes each) at room temperature. If the antibody is conjugated to a fluorescent tag, then the incubation and washing steps should be performed at dark (Note 4).
6. **Mounting:** During the mounting procedure, the embryos should be placed in a small volume (20 µl) of mounting medium in the circle on the slide (Figure 4) (Note 5). First, place the mounting or anti-fade medium in the circle. Then, transfer the embryos into the middle of medium. Eyelash probe could be used to move embryos into the middle of circle. Let the embryos sink to the bottom of the medium. Place a coverslip very carefully on the circle on the slide, trying not to move embryos toward the edges of the circle. Seal around the edges of coverslip with nail polish. The mounted embryos can be examined right away or stored at 4°C.

Fig. 4. Making circles of nail polish on the slide for mounting of immunostained embryos.

7. **Microscopic examination:** Depending to the type of secondary antibody used and the available equipment, the embryos can be imaged using light, epi-fluorescence, or confocal laser scanning fluorescence microscopy.

3.3 Notes

Note 1. Male mice reach sexual maturity at the age of 8 weeks. It is important that after weaning the individual male pups to be kept in separate cages. It is believed that keeping several male pups together in one cage, except in the dominant male, may suppress their hormonal maturity. It is also important to place one injected female in the male's cage. Male mouse should not be placed in female's cage. Only one female and not more should be placed in the male's cage. The day after mating males and females should be separated again.

Note 2. Permeabilization is only necessary when an intracellular antigen or protein is to be detected. For immunocytochemical detection of proteins or antigens which are localized on the cellular membrane, a permeabilization step is not performed.

Note 3. Permeabilization and incubation of embryos in primary antibody causes them to sink toward and occasionally adhere the bottom of depression slides. This makes the transfer of embryos between different containers very difficult. Eyelash probe (commercially supplied or homemade by gluing an eyelash to a needle) would be an indispensible device for these situations. With this device under a stereomicroscope, it would be very easy to detach the embryos from the bottom of depression slides and guide them toward the transfer pipette.

Note 4. Different secondary antibodies may be used. If the secondary antibody is conjugated to biotin, alkaline phosphatase, or horseradish peroxidase, different substrates are used to reveal antigen-primary antibody-secondary antibody complexes and different procedures are followed before the mounting step.

Note 5. Placing coverslip directly onto a slide with embryos in between will cause the physical rupture and burst of embryos. Thus, it is very important to produce a space between slide and coverslip. For this purpose, small circles (with a diameter of 5mm) are made on the slide by nail polish. We use an insulin syringe (attached to its needle) filled with nail polish to make the circles with defined edges. When the circle of nail polish is dried, the space in the middle will be used for mounting of embryos.

4. Conclusion

The mammalian preimplantation development contains a highly regulated series of cellular and molecular events that are necessary for normal cell growth, cell division and differentiation. Our understanding of the mechanisms involved in these events has significantly increased in recent years, while much remains to be learned about the mechanisms involved in controlling growth and proliferation, transcriptional control and cell fate decisions. Immunocytochemistry has had and remains to have a significant role for the discovery of these events. In this chapter, its contribution to our current understanding of the different aspects of preimplantation development has succinctly reviewed. In addition, the ICC procedure has been elaborated.

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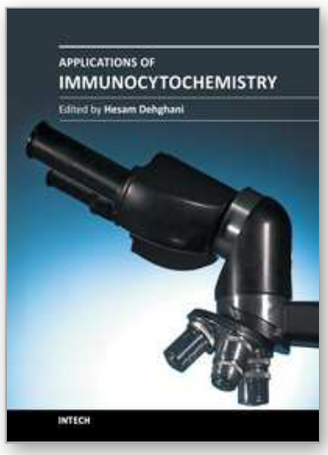

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Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. However, over the years many aspects of this procedure have evolved within a plethora of experimental setups. There are different ways to prepare a given specimen, different kinds of antibodies to apply, different techniques for imaging, and different methods of analyzing the data. In this book, various ways of performing each individual step of immunocytochemistry in different cellular contexts are exemplified and discussed. Applications of Immunocytochemistry offers technical and background information on different steps of immunocytochemistry and presents the application of this technique and its adaptations in cell lines, neural tissue, pancreatic tissue, sputum cells, sperm cells, preimplantation embryo, arabidopsis, fish gonads, and Leishmania.

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