# Methodology for Detection of Spindle-associated Proteins by Confocal Microscopy in Mammalian Oocytes*

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**Abstract** The mammalian oocytes are important materials for study of meiotic cell cycle in developmental biology and cell biology. Confocal microscopy is a powerful technique in detecting the spindle-associated proteins in oocytes. However, in order to obtain the ideal results, some technological modifications should be made according to the characteristics of mammalian oocytes. Among them, the oocyte preparation, fixation, membrane permeabilization, fat extraction, and antibody incubation are the key steps. Furthermore, the differences among oocytes from various mammalian species should be considered in the actual manipulation of confocal microscopy.

**Key words** oocyte, mammal, confocal microscopy, spindle

The progression of meiotic cell cycles is accompanied by extensive microtubule re-organization. The assembly, rotation, and elongation of meiotic spindles are crucial for the correct separation of chromosomes, which guarantees the stability of genomes during reproduction[1]. The spindle microtubules themselves are the polymers of α- and β-tubulin, but their assembly/disassembly and functions are regulated by numerous microtubule-associated proteins, especially several important protein kinases (For review, see Fan *et al.*[2]). It was reported by others and us recently that mitogen-activated protein kinase (MAPK)[3, 4], polo-like kinase[5, 6], and 90 ku ribosome S6 protein kinase (p90rsk)[7, 8] play key roles in the regulation of meiotic cell cycles in mammalian oocytes. All these kinases are localized to the spindle poles or equator region at certain developmental stages. But their downstream molecules on spindle or the regulatory mechanism of their distribution are scarcely known. The spindle-associated protein kinases may interact with microtubule-nucleating proteins at microtubule-organizing center (MTOC), such as γ-tubulin, or motor proteins along the microtubules.

When compared to somatic cells, the mammalian oocytes are larger in size but smaller in number. To study the spatial relationship between spindle and its regulatory proteins, new and special methodology must be employed. In the last five years, laser scanning confocal microscopy was extensively used in the study of meiotic cell cycle regulation. The combination of light microscopy and electronic techniques allowed the researchers to view the delicate spatial images in the cells, so as to provide concrete information regarding to the subcellular localization of specific proteins[9]. In this study, using mouse and pig oocytes, we optimized the methodology of confocal microscopy in studying spindle-associated proteins in mammalian oocytes, and provide the simple but effective protocols that are easy to follow by other researchers.

## 1 Materials and methods

### 1.1 Collection and culture of oocytes

Fully grown germinal vesicle-intact mouse oocytes were collected from ovaries of 4 ~ 6 week-old Kunming mice 48 h after the females were injected with 10 IU pregnant mare's serum gonadotrophin (PMSG). Cumulus-free and GV-intact oocytes were released from antral follicles by puncturing the follicles with a needle in M2 medium (Sigma) supplemented with 60 mg/L penicillin and 50 mg/L streptomycin. All cultures were carried out in M2 medium at 37℃ in a humidified atmosphere of 5% $CO_2$.

Metaphase Ⅱ-arrested eggs were obtained from mice of the same strain. Females were superovulated by intraperitoneal injection of 10 IU of PMSG, and 48 h later, they were injected with 10 IU of human chorionic gonadotrophin (hCG). Mice were sacrificed and oviducts were removed at 15 h post-hCG injection. Using a pair of fine forceps to tear the oviducts, cumulus masses were collected in M2 medium. The cumulus cells surrounding the eggs were removed by a brief exposure to 300 IU/ml hyaluronidase and repeated pipetting, followed by three washes in M2 medium.

Pig ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 1 h. Oocytes were aspirated from antral follicles (2 ~

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6 mm in diameter) with an 18-gauge needle fixed to a 20 ml disposable syringe. After washing three times with maturation medium (see below), oocytes with a compact cumulus and evenly granulated ooplasm were selected for maturation culture. The medium used for maturation culture was improved TCM-199 (Gibco, Grand Island, NY) supplemented with 75 mg/L potassium penicillin G, 50 mg/L streptomycin sulphate, 0.57 mmol/L cystein, 0.5 mg/L FSH, 0.5 mg/L LH and 10 μg/L epidermal growth factor (EGF). A group of 20 oocytes was cultured in a 100 μl drop of maturation medium for up to 46 h at 38.8℃ in an atmosphere of 5% $CO_2$ and saturated humidity.

### 1.2 Confocal microscopy

#### 1.2.1 Mouse oocytes

After removal of ZP in acidified Tyrode's solution (pH 2.5), oocytes were fixed in 3% formaldehyde, 2% sucrose in PBS for 30 min at room temperature and then incubated in incubation buffer (0.5% Triton X-100 in 20 mmol/L Hepes, pH 7.4, 3 mmol/L $MgCl_2$, 50 mmol/L NaCl, 300 mmol/L sucrose, 0.02% $NaN_3$) for 30 min. After placed in methanol for 5 min at −20℃, the eggs were washed in PBS with 0.1% Tween 20 and 0.01% Triton X-100 (washing solution) for three times and then incubated with 1∶100 diluted monoclonal mouse anti-Plk (Zymed Laboratories Inc., South San Francisco, CA) or polyclonal mouse anti-rabbit ERK2 for 1 h. The eggs were rinsed three times with washing solution and incubated with 1∶100 FITC-conjugated goat anti-mouse IgG for 45 min, followed by a 10 min staining with 10 mg/L propidium iodide (PI). Finally, the eggs were mounted between a coverslip and a glass slide supported by four columns of a mixture of Vaseline and paraffin (9∶1). The slides were sealed with nail polish and examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems).

In another option, ZP-intact mouse oocytes were fixed by 4% paraformaldehyde in PBS for 30 min at room temperature, and then permeabilized in incubation buffer. After blocked by 1% BSA in washing solution, the cells were incubated with 1∶100 diluted polyclonal rabbit-anti p90rsk, or 1∶400 diluted polyclonal rabbit anti-γ-tubulin (Sigma) for 1 h. The steps of washing, second antibody incubation, and PI staining were just the same as described above.

In some experiments, the spindle organization of oocytes was determined by incubating the oocytes in 1∶50 diluted FITC-anti-α-tubulin (Sigma) for 1 h after fixation and permeabilization was conducted as described above. For double staining of γ-tubulin and α-tubulin, oocytes fixed with 4% paraformaldehyde were sequentially incubated with rabbit anti-γ-tubulin antibody, TRITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, INC, PA), and FITC-conjugated anti-α-tubulin anitibody. Nonspecific staining was determined by substituting primary antibodies with normal rabbit IgG.

#### 1.2.2 Pig oocytes

After removing the zona pellucida in acidified Tyrode's medium (pH 2.5), oocytes were fixed with 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. Cells were permeabilized with PBS containing 1% Triton X-100 overnight at 37℃, followed by blocking in 1% BSA for 1 h and incubation overnight at 4℃ with mouse anti-Plk1 antibody, rabbit anti-p90rsk antibody, or rabbit anti-ERK2 antibody diluted 1∶100 in blocking solution. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100 (washing solution) for 5 min each, the eggs were labeled with FITC-conjugated goat anti-rabbit IgG diluted 1∶100. Nuclear status of oocytes was evaluated by staining with 10 mg/L propidium iodide (PI) for 10 min. Following extensive washing, samples were mounted as described above.

For staining of meiotic spindles, fixed oocytes were incubated with FITC-conjugated anti-α-tubulin anitibody (Sigma) for 1 h. Then the cells were stained with PI and sealed as mentioned above. Nonspecific staining was determined by substituting primary antibodies with normal rabbit IgG. Each experiment was repeated 3 times and at least 20 oocytes were examined each time.

## 2 Results

### 2.1 Detection of spindle-associated proteins in mouse oocytes

As shown in Figure 1, meiotic spindle was organized in mouse oocytes at MⅠ (Figure 1a) and MⅡ (Figure 1c) stages, with chromosomes at the equator of the spindle. γ-tubulin was localized at the spindle poles in MⅡ-arrested mouse oocytes (Figure 1b). The structural association between γ-tubulin and α-tubulin was shown by double-staining of these two proteins in mouse oocytes at MⅡ stage (Figure 1c). ERK2 was detected on the whole spindle, but a strong staining was observed at the poles of meiotic spindle (Figure 1d). In mouse oocytes at MⅠ stage, p90rsk was also localized to the spindle poles, and a weak staining of this protein was also observed at the equator of meiotic spindles (Figure 1e). Fertilized mouse oocytes released the second polar body at approximately 2 h after insemination. In these cells, Plk1 was localized to the cytoplasmic connection between the separating chromosomes, putatively the position of contractile ring (Figure 1f). In negative control experiments in which the first antibodies were substituted by normal rabbit or mouse IgG, no specific fluorescent staining was detected in oocytes at any developmental stages (data not shown).

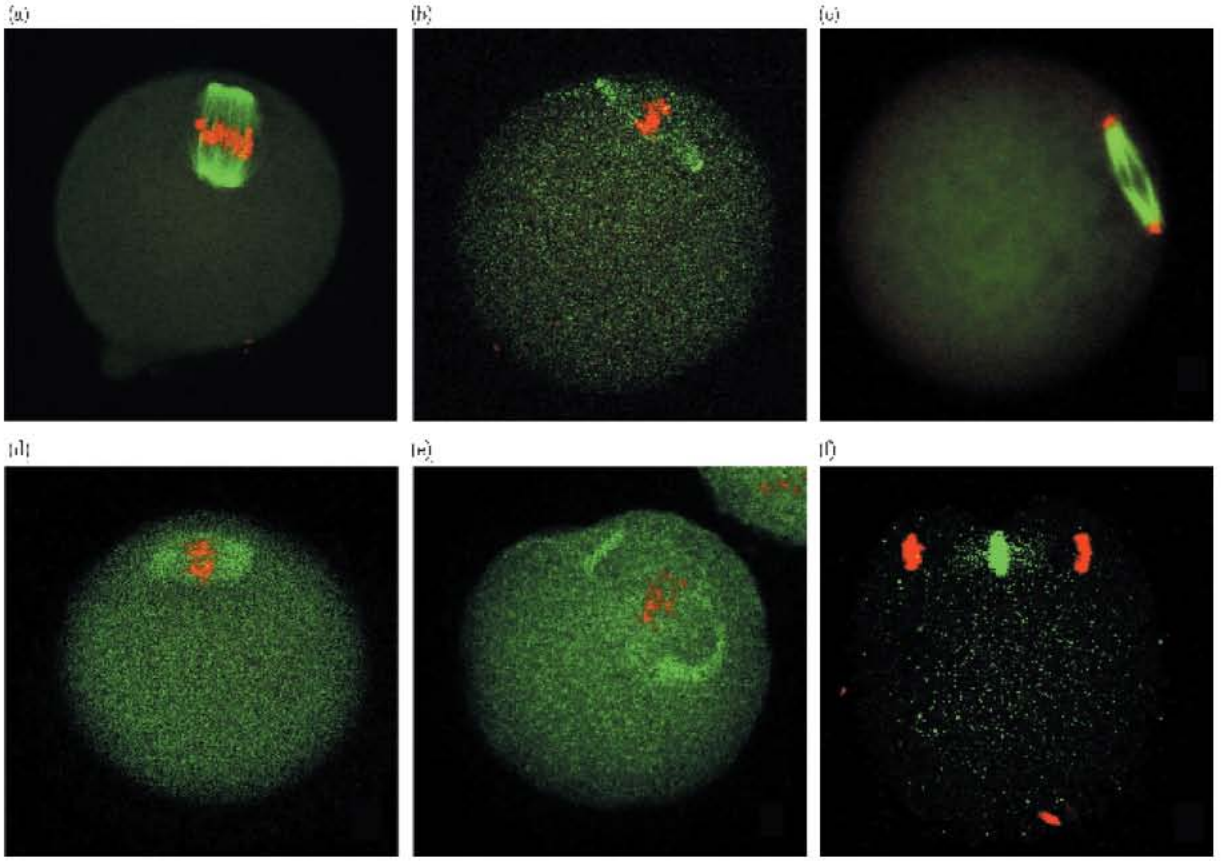

**Fig. 1　Confocal microscopy for spindle-associated proteins in mouse oocytes**

(a) Staining of α-tubulin in a mouse oocyte at MⅠ stage; (b) Localization of γ-tubulin in an MⅡ-arrested mouse oocyte; (c) Double staining of α-tubulin (stained in green) and γ-tubulin (stained in red) in an MⅡ-arrested mouse oocyte; (d) Localization of ERK2 in an MⅡ-arrested mouse oocyte; (e) Localization of p90rsk in a mouse oocyte at MⅠ stage; (f) Detection of Plk1 in a mouse zygote 2 h after *in vitro* fertilization.

## 2.2　Detection of spindle-associated proteins in pig eggs

As shown in Figure 2, meiotic spindle was organized in pig oocytes at MⅠ (Figure 2a) and MⅡ (data not shown) stages. But when compared to the mouse meiotic spindle (Figure 1a), the meiotic spindle in pig oocytes is much smaller and barrel-shaped. In pig oocytes at anaphase Ⅰ, strong staining of REK2 was detected at the area between the separating chromosomes, presumably the location of elongating anaphase spindle (Figure 2b). As shown in mouse oocytes (Figure 1e), p90rsk was localized at the spindle poles of pig oocytes at MⅠ stage (Figure 2c). During the emission of the first polar body, Plk1 was detected at the area between the separating chromosomes, putatively the position of spindle middle plate at anaphase (Figure 2d).

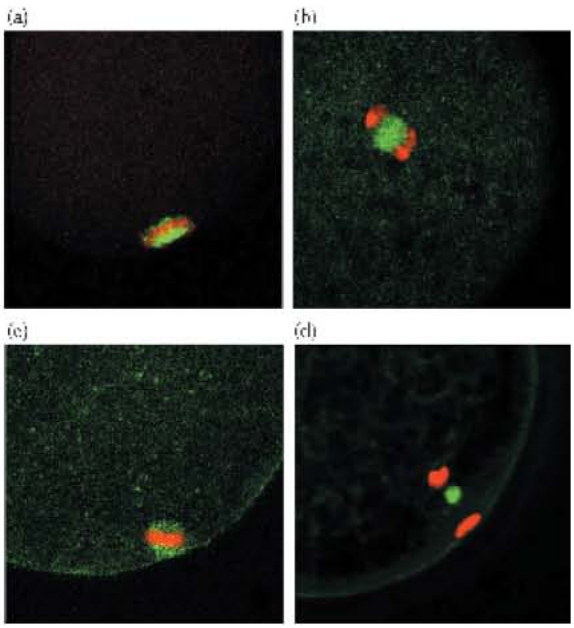

**Fig. 2　Confocal microscopy for spindle-associated proteins in pig oocytes**

(a) staining of α-tubulin in a pig oocyte at MⅠ stage; (b) localization of REK2 in a pig oocyte at anaphase Ⅰ; (c) staining of p90rsk in an MⅡ-arrested pig oocyte; (d) localization of Plk1 in a pig oocyte at anaphase Ⅰ.

## 3 Discussion

In this study, several important spindle-associated proteins were detected at different locations of meiotic spindle by confocal microscopy, both in mouse oocytes and in pig oocytes. The results showed that the confocal microscopy methods employed in this study are sensitive and specific. During our former experiments, a set of efficient methods for the study of spindle-associated proteins by confocal microscope was established, and many methodological approaches were developed to optimize the ultimate results obtained.

Firstly, the oocytes must be perfectly prepared before fixation. Mammalian oocytes are enclosed by cumulus cells, which must be separated from the oocytes before further steps of confocal microscopy. The cumulus cells of mature pig or mouse oocytes at M Ⅱ stage could be removed by exposure to 300 U/ml hyaluronidase and repeated pipetting. However, the cumulus cells around the immature oocytes are more difficult to be removed. Mouse oocytes at GV stage could be freed of cumulus cells by repeated pipetting with a fine Pasteur pipette, whose diameter is a little smaller than the oocytes. Pig oocytes with cumulus cells could be vortexed in an eppendoff tube with hyaluronidase at a high concentration until the cumulus cells were removed. According to some reports, it is not necessary to remove the zona pellucida (ZP) during confocal microscopy[10], however, we found here in our experiments that the existence of the ZP could prevent the free defuse of antibody, and result in a high background during confocal detection. Thus, we recommend remove the ZP by a very short exposure (just several second) of oocytes to acidic M2 (pH 2.5) before fixation.

Secondly, appropriate fixation methods are determinative to the final results. The most popular fixative reagent used in immunofluorescense is paraformaldehyde diluted in PBS at the concentration of 3% ~4%. The ZP of mouse oocytes can be destroyed after paraformaldehyde fixation, so it is unnecessary to dissolve the ZP using acidic M2 medium if the fixation reagent is paraformaldehyde. At least 30 min is necessary for the samples to be adequately fixed by paraformaldehyde, but according to our experience, the fixation time up to 1 h is not harmful to the final results. Paraformaldehyde is unstable in water solution, so it must be made freshly before use. However, paraformaldehyde is not always the best choice of fixation. For example, 3% formaldehyde is better than 4% paraformaldehyde in detecting Plk1 in mouse oocytes. And a further fixation step in methanol at −20℃ for 5 min may improve the structure integrity of microtubules in mouse oocytes[5].

The cell membrane is impermeable to large protein molecules including immunoglobulin. So the intact cells must be permeabilized by detergents before antibody incubation. The ideal detergents used in confocal microscopy are Triton X-100 and Tween 20. Organic reagents such as methanol, ethanol and acetone can also increase the membrane permeability when they are used as fixtives. As we reported before, 0.1% Triton X-100 treatment of 15 min is enough for the confocal detection of protein kinase C, which exists in the cytoplasm or on the membrane. However, for sensitive detection of spindle-bounded proteins, higher concentrations of Triton X-100 and longer incubation time are necessary. The cytoskeleton-associated proteins could resist severe detergent treatment and present prominent expression signal after 0.5% Triton X-100 treatment for 30 min, as shown in Figure 1.

Although many similarities exist among oocytes of various mammalian species, there are also some important differences when comparing the oocytes from different species. In this study, the methods of confocal microscopy for rodents (mouse) and farm animals (pig) are compared. The pig oocytes are larger than mouse oocytes (120 μm vs. 70 μm in diameter), and are abundant in fat droplets[4]. The mouse oocytes are transparent, while the pig oocytes are opaque. Furthermore, the meiotic spindle of pig oocytes is much smaller than that of mouse oocytes. So it is more difficult to detect spindle-associated proteins in pig oocytes than in mouse oocytes. To solve these difficulties, we increased the incubation time and concentration of Triton X-100 during permeabilization. As reported by us, an overnight treatment with 1% Triton X-100 at 37℃ is necessary to extract the fat in the cytoplasm of pig oocytes before antibody incubation. Samples were incubated overnight at 4℃ to facilitate the specific binding of antibody. It is also necessary to point out that, differing from mouse oocytes, the zona pellucida of pig oocytes is resistant to 4% paraformaldehyde, and the ZP must be removed by acidic M2 before fixation.

According to our experience, the samples prepared using the methods reported here could be stored at −20℃ in a dark box for more than one week without influencing the laser scanning results. Overall, the confocal microscopy for mammalian oocytes is a very flexible technique. Based on the principles introduced above, one needs to adjust and optimize the detail steps to obtain satisfactory results.

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# 用激光共聚焦显微术检测哺乳动物卵母细胞中纺锤体相关蛋白的方法*

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摘要 哺乳动物卵母细胞是发育生物学和细胞生物学的重要研究对象. 激光共聚焦显微术是检测卵母细胞中纺锤体相关蛋白的有用技术，但是针对哺乳动物卵母细胞的特点需要做许多调整以得到理想结果. 其中，卵母细胞的准备、固定、透膜、脂类抽提、抗体孵育步骤十分关键. 另外，不同物种的卵母细胞也有差异，在进行激光共聚焦研究时需要做出相应的调整.

关键词 卵母细胞，哺乳动物，激光共聚焦显微术，纺锤体
学科分类号 Q26

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