Chapter 4

4.0 EXPERIMENT 1

TITLE: OPTIMISATION OF THE ICC PROCEDURES

4.1 ABSTRACT

Experiment 1 was conducted with the main objective to establish ICC technique for morphological analysis of the mouse oocytes and embryos in local laboratory. Several tests were conducted to achieve specific aims which included; a) to study efficacy of the available instruments and system for detecting fluorescence signals from mouse oocytes, b) to compare efficiency of improved methods to reveal oocyte morphology. Fluorescence images of the meiotic spindles, cortical granules and nuclear materials were compared between sample preparations methods. The results showed that the methods employed were reliable to be used for detecting the meiotic spindle, cortical granules and nuclear materials. The microdroplet method used in staining procedure produced high number of total oocytes recovered as compared with poly-l-lysine coated cover slip method. Meanwhile, the appearance of meiotic spindle in oocytes was significantly affected by the method used during samples preparation. A significantly higher percentage of clear meiotic spindles images were obtained from zona intact and methanol fixed oocytes, which were 62.8% and 64.7%, respectively ($p<0.05$). However, the images for cortical granules and nuclear materials were not significantly affected. The optimum range of titres of the fluorescent dyes used was equivalent to those reported by many laboratories. The non-specificity of the emission spectrum was detected in the present microscopy system. This was evidenced by images having both blue and green dyes overlapping each other, termed as cross-talk under the UV excitation filter. Hence, the quality of images obtained in fluorescence microscopy

depends greatly upon the methods employed in sample preparation and the system utilised for acquisition of images of the oocytes.

Keywords: ICC procedures, Fluorescent signals, emission spectrum, cross-talk

4.2 INTRODUCTION

Immunocytochemistry (ICC) refers to the method used for the detection of cell constituents by tagging a specific cellular antigen usually proteins to a fluorescent antibody. This technique is the window to advanced studies in cellular architectures and functions and is the method of choice in many laboratories ever since it was first established almost a century ago. The technique attracted attention of many more researchers when Coons and Kaplan in early 1950s introduced the labeling technique of antibodies with a fluorescence dye or fluorophore in the study of antibody-antigen interactions. This has profoundly changed the field of cell study. This was followed in 1994 by the discovery that really brought fluorescence microscopy to the forefront came, when the now-famous green fluorescent protein (GFP) is used to express cellular processes in living organisms. This was a landmark evolution in the field of cell biology.

Although in principle, the method is expected to be very specific with high precision (Glass, 1971; Vonesh *et al*., 2007), however, one may experience difficulties to identify the structures under study should other elements such as artifacts, autofluorescence, non-specificity and co-localisation had intervened with the image produced. Most importantly improper specimen preparation may impede or block antigen labeling in the cell structures too. The main objective to be accomplished in ICC and fluorescence microscopy techniques is the ability to distinguish the real structures of interest which will solely fluoresce from the sample under specified

microscope filter. Thus, it is very important to have sets of control samples in order to contrast cell structures from any foreign body or microscope artifact.

Variation in the laboratory protocols of the technique is well reported. Many improvements have been conducted in ICC protocols to yield best possible results from the staining technique. Due to several reasons and constraints, an exact similar protocol was hardly practised between laboratories. However, the basic principle of ICC concepts is clearly applied in each procedure. Some of the contributing factors to the existence of different ICC protocols are the nature of samples, the amino acid components of the proteins under study, the fixative used, the specificity of antibody and instrumentation. Also, varying concentrations of the solvents used for fixation and permeabilisation, the different grades of instruments, the sensitivity of antibody and last but not least the skill and experiences of the personnel involved are accounted for in determining the end results.

List of protocols in conducting ICC procedures in mammalian gametes is summarised in Table 4.1. In brief, extensive work using ICC procedures proliferates since the nineteenth century and continued until today. Several steps which ought to be closely observed while conducting the staining procedures are sample preparation, fixation, permeabilisation, antibody-labeling, mounting and image analysis. It is very unlikely to have a similar ICC protocol being practised between laboratories due to variation of samples and laboratory conditions. In general, ICC of oocytes or embryos requires preliminary steps of sample preparation such as zona dissolution and stabilisation of the structure of interest especially microtubules and microfilaments. Common procedures in oocyte preparation practised across many laboratories were denuding of oocytes using hyaluronidase (0.1%) and pronase (0.5%) (Schatten *et al*., 1985), followed by dissolution of zona using acid Tyrode's (Sutovsky *et al.,* 1996*)* or

acidified TALP-Hepes (pH 2.5) with BSA (0.3%) (Meng and Wolf, 1997) and finally attach them onto a poly-l-lysine coated coverslip. The zona layer which surrounds an oocyte or embryo may impede antibody binding and fluorophore absorption to tag oocyte structure and eventually diminishing the quality of end results. Adequate permeabilisation step is required to ease antigen-antibody labeling. This is accomplished by incubating oocytes and embryos in detergent such as Triton X-100 (1%) which disrupts membrane polarity, hence, allows easy penetration of antibodies and fluorophores into the oocytes. Triton X-100 may cause membrane extraction and a milder detergent such as saponin (0.5%) is an option for permeabilisation that leaves cells structurally intact (Anderson, 2008).

Common fixatives used to fix oocytes or embryos in ICC technique are formaldehyde (Schatten *et al*., 1983), paraformaldehyde (Patricia, 1983), methanol (Tae Do, 2002), cold methanol (Schatten *et al*., 1992) and microtubule-stabilising buffer (Hiroyuki, 1997). However, no specific comparative analysis is mentioned on the effects of using different fixatives on fluorescence images produced. Nevertheless, it was reported that prolonged fixation period especially in aldehyde solvents may mask antigenic determinants due to aldehyde cross-linking and increased hydrophobicity of sample which cause diffused staining (Schatten *et al*., 1983; EMD-Calbiochem, 2008). Culture medium and some fixatives, for example, glutaraldehyde may induce autofluorescence of the sample and background staining (Garsha, 2008), which would eventually results in reduced specificity and contrast, hence, noisy background appears in the image taken.

Many protocols have given the emphasis on the importance of using blocking solution to reduce non-specificity of antigen-antibody binding or cross-reactivity. Non-specific binding occurs when an antibody elicited by one antigen cross-reacts with

unrelated antigens (Garsha, 2008). There are several substances commonly used as blocking agents such as normal human serum (Patricia, 1983), goat serum and milk powder (Hiroyuki *et al*., 1997), and glycine with bovine serum albumin (BSA) (Simerly *et al.*, 2002) in varying concentrations. It is best to use serum from the species of the source of second antibody, although, BSA is the commonest used. Gelatin or serum other than the species of primary antibody may also be used to block non-specific binding too (EMD-Calbiochem, 2008). For research purposes, monoclonal antibodies are preferable because they are well characterised, an important consideration for optimising immunolabeling (Garsha, 2008).

Most commonly, immunofluorescence employs two sets of antibodies; a primary antibody which is used against the antigen of interest and a subsequent dye-coupled secondary antibody which recognises the primary antibody. This protocol is known as indirect labeling which is believed to provide amplified signal. Typically this is accomplished by using antibodies obtained in different species. For example, antibody from rabbit is used to immunise a goat which produces anti-rabbit antibody. The anti-rabbit antibody is then used as a reagent to detect the binding of mouse antibody which fluoresces when tagged using a fluorescent dye-coupled antibody. The study on oocytes or embryos microtubules architecture usually employed primary antibody which is the monoclonal anti-β tubulin or E7 to label its tubulin component. The concentration of E7 used and the duration of antigen-antibody incubation period were never similar between laboratories. The shortest incubation time reported was 40 minutes (Simerly *et al*, 1993; 2003) and the longest was 24 hours (Hiroyuki *et al*., 2002). Antibody E7-antigen complex are then tagged with fluorescent dye-coupled secondary antibody such as FITC-goat anti mouse IgG. This indirect staining technique has produced high precision of end results.

On the other hand, it is advantageous to use primary antibodies which are directly labeled with a fluorophore as it decreases the steps in the staining procedures. This is known as direct staining. A different range of fluorophores has been used to stain oocyte structures. Among the commonest are Hoechst, DAPI or Propidium iodide (PI) to stain nuclear materials; while FITC or TRITC conjugated to LCA to reveal cortical granules of oocytes. However, a more highly specific result can be obtained when protein of a particular structure such as tubulin of meiotic spindle is first tagged by anti-tubulin antibody followed by either FITC or TRITC conjugated to anti-tubulin through indirect staining procedure in contrast to a direct staining method such as Hoecsht or DAPI staining.

Fluorescence dyes or fluorophores are sensitive to light and easily bleached upon exposure to high intensity of light and prolonged time-span. Loss of activity which is caused by photo-bleaching, however, can be controlled through several means such as by reducing the intensity or time-span of light exposure, by increasing the concentration of fluorophores or by employing more robust fluorophores that are less prone to bleaching. Adding anti-fading agent into the mounting medium before the oocytes or embryos are finally mounted onto coverslips has also been recommended. Some of the anti-fade agents commonly used are Vectashield and DABCO. In addition, prepared slides are generally covered using aluminum foil and stored at $4^{o}C$, although immediate observation of the prepared samples under the fluorescent microscopy is highly recommended. Table 4.1 shows variation in ICC procedures practised by several laboratories for staining oocytes and embryos.

Table 4.1: Variations in ICC practices and procedures for staining oocytes and embryos

Year	Author	Cellular structure	Procedure	Description
1983	Patricia *et al.*	Centrosome, Nuclear materials	Fixation	Prefixed in Parafomaldehyde (2%) and post-fixed in methanol
			Blocking solution	Normal human serum
			Mounting medium	Glycerol-phosphate buffer (1:5)
			Staining	Add Hoechst into mounting medium
1985	Schatten *et al.*	Microtubules	Fixation	Microtubule stabilization buffer
			Removal of zona	Hyaluronidase (0.1%) and Pronase (0.5%)
1991	Stearns *et al.*	Centrosome (δ-tubulin)	Staining for microtubules	Primary antibody (E7) Anti-δ tubulin rabbit polyclonal antibody (1:100)
1995	Simerly *et al.*	Oocyte cytoskeleton	Fixation	Formaldehyde
			Permeabilization	Triton x-100 (1%) for 40 minutes
			Mounting	Vectashield
1996	Sutovsky *et al.*	Oocyte cytoskeleton, nuclear materials	Removal of zona	Acid Tyrode's solution
			Oocyte preparation	Poly-l-lysine coated coverslips
			Fixation	Formaldehyde (2%), 10 mins
			Permeabilization	Triton x-100 (1%), 40 mins
			Staining for the cytoskeletal	Primary antibody: anti-β tubulin mouse monoclonal (E7) Dilution 1: 10- 1 hr incubation Secondary antibody: FITC – conjugated goat anti mouse IgG Dilution 1:40, 1 hour incubation
			Staining for the nuclear materials	DAPI (5mg/ml)-Penultimate rinse
			Washing	Triton x-1000 (1%) in PBS
			Mounting	Vectashield
			Zona removal	Acidified TALP-HEPES and BSA (0.3%, pH 2.5)
			Oocyte attachment	Poly-l-Lysine coated coverslip overlaid with Ca^{2+}, Mg^{2+} free PBS (2-3 mins, 37°C)
1997	Meng and Wolf	Oocyte microtubule and DNA	Fixation	Paraformaldehyde (2%)
			Permeabilization	Triton x-100 (0.1%), 30 mins or overnight
			Blocking solution	PBS: Triton x-100 (0.1%): BSA (0.3%): Glycine (150mM) PBS:
			Washing solution	Triton x-100 (0.1%): BSA (0.3%)
			Staining for microtubule	Primary antibody: IgG monoclonal antibody to β-tubulin (1:200, 40 mins) Secondary antibody: FITC-conjugated goat anti mouse IgG (1:32), 40 mins

			Staining for DNA Mounting solution	Hoechst (10 ug/ml, 10 mins) Glycerol (50%) and Slow Fade solution, sealed with nail polish
2002	Hiroyuki *et al.*	Cytoskeleton of oocytes	Fixation Blocking solution and washing medium Staining for microtubule Staining for microfilament Staining for DNA Mounting medium	Microtubule stabilising buffer, 1 hour Calcium-free DPBS: BSA (2%), goat serum (2%): milk powder (0.2%): sodium azide (0.2%): Triton x-100 (0.1%) Primary antibody: Anti- β tubulin (1:200, 4^{o}C), overnight FITC-conjugated (1:200, 37^{o}C), 2hr+ Rhodamine-phalloidin (1:1000), 1 hour Hoechst (10 ug/ml) PBS:Glycerol (1:1)
2002	Tae Do	Microtubule and chromatin organization in bovine oocytes	Fixation Post-fixation incubation Staining for microtubules DNA staining	Methanol after permeabilization PBS, sodium azide (0.02%), BSA (0.1%) 4-well dish Primary antibody: Monoclonal anti-α tubulin (1:200) FITC-labelled anti-mouse IgG Propidium iodide (5 µg/ml)
2003	Simerly *et al.*	Microtubules and microfilaments of oocytes	Fixation Oocyte attachment Permeabilization Blocking solution Staining for microfilaments Staining for microtubules DNA	Cold methanol (10 min) and formaldehyde (2%)- 24 hours Poly-l-lysine coated coverslip Glycerol: Triton x-100 (1%): methanol (10%) or PBS (10mM): Triton x-100 (1%), 40 mins PBS: glycine (50mM): BSA(3mg/ml), 30 mins Rhodamine-phalloidin (15 U/ml), 30 mins Primary antibody: Anti-β tubulin (E7, 1:5), 60 mins Secondary antibody: FITC-conjugated anti-mouse IgG (1:40), 60 mins Hoechst (10 ug/ml, penultimate rinse)

One of the most challenging elements while carrying out conventional ICC procedures using oocytes and embryos was high incidence of cell loss. The facts that oocytes and embryos are round and not flat as compared to any other cells had imposed a considerable challenge while carrying this procedure initially. Thus, the aims of this experiment were to conduct preliminary testing on the application of conventional ICC method and to compare the images of oocyte produced with that from improvised methods. Sensitivity and specificity of the methods were looked at; whereby sensitivity refers to how effective the probes can stain the specific structure at a very low concentration, while specificity defines the precise cellular structures labeled by the specific dyes.

Optimisation of ICC procedures in this study included the followings:

a) Optimisation of titre concentrations of the antibody and fluorophores The optimal titres of both primary and secondary antibody for tubulin as well as other fluorophores which included rhodamine and Hoechst is the key to ICC staining procedure. It is crucially important to avoid over-staining which results in over-expression of the cellular structures that may lead to non-specificity and diffused staining. Also, the optimal dilution could prevent overuse of the antibody and fluorophores, which are generally very costly and not easily obtained in local market.

b) Preparing controls The aim of this experiment was to identify the actual fluorescence image produced when a single probe is used to tag a specified cellular structure and viewed using a set range of excitation-emission filter. This was also to determine specificity effect of the probe used. It has been recommended that when multiple dyes are used on the same sample, a preliminary testing is to be conducted on the effect of one fluorophore on the structure of interest. This is to avoid confusion over the identification of a labeled structure when more than one colours fluoresced at one

excitation-emission wavelength and in worse scenario the colours overlap within the same location of the labeled structures. This staining error is known as cross-talk. Cross-talk appeared when two or more dyes excite and fluoresce simultaneously under one or more excitation-emission spectrum of the fluorescent filters due to having similar emission spectra between two or more fluorochromes. This may lead to difficulty for identification purpose of the cell structure. A series of sample preparation was utilised to distinguish the images as indicated in Table 4.2. This was a modification of protocol described by Lewitt and Pitt (2002).

c) Sample preparation methods Cells can be prepared for ICC staining using several methods. Two tests were conducted on sample preparation, which were the effect of sample preparation on cell recovery and the effect of fluorescence staining on zona intact and zonaless oocytes. The conventional procedure usually adheres cells onto the coverslips prior to staining and this is a crucial step failing which it may cause sample loss. Meanwhile, oocyte is enclosed by a non-living layer called the zona pellucida which may impede antigen-antibody binding. Thus, many protocols require removal of zona prior to staining the oocytes using acid Tyrode's solution. The quality of the fluorescence images produced from zona intact and zonaless images were compared.

d) Effect of fixatives on the fluorescence images This test was conducted with the aim to compare images of the oocyte structures produced after it was fixed in two fixatives which were formaldehyde and methanol.

4.3 MATERIALS

The chemicals and reagents used in this experiment were as listed in Section 3.8.4**.** In brief, the first antibody used to label the microtubule was a monoclonal anti-β tubulin

antibody (E7) and the second antibody was flourescein isothiocynate (FITC) goat anti-mouse IgG (KPL 02-18-06). The fluorophore for the cortical granules was rhodamine *Lens culinaris* (lentil seeds) agglutinin (RL-1042, LCA-Vector laboratory) and Hoechst for staining the nuclear materials. Alexa Fluor® 568-rhodamine-phalloidin (Molecular Probes –A 12380, 300 U) was used to label F-actin filaments of the mouse oocytes. The concentrations for each probe and the antibody prepared were based on the descriptions given by the respective manufacturers and previous reports. The sample used in this experiment was the mouse oocytes.

4.4 PROCEDURES

The following sections described four main procedures included in the experiment. There were optimisation of titre concentrations, preparation of control samples, oocyte preparation methods and effect of fixatives on fluorescence images of oocytes.

4.4.1 Optimisation of Titre Concentrations of Antibody and Fluorescent Probes

Serial dilution of each antibody and the fluorescent dyes (fluorophores) were prepared as described in the product statement by the respective manufacturers. The range of concentrations for the fluorescent dyes was 5-20 µg/ml and the concentrations for the primary and the secondary antibodies were between 1:10 and 1:20, respectively. Antibody preparation was as described in Section 3.8.5.1.2. For staining purpose, 50-100 µl of the aliquots was used and incubated with approximately 5 to 10 oocytes at each time. The ICC staining procedure was described in Section 3.8.

4.4.2 Preparing Control Samples

Oocytes were obtained as described in Section 3.6.5.2. A series of control slides for determining the reactivity of the antibody and the fluorophores was prepared as below. In order to visualise a tagged structure to fluoresce, a negative control was prepared by simply omitting the specified fluorophore from the staining protocols. This step is highly recommended especially when multiple dyes are used to label more than one oocyte structures on the same sample. Each slide was then observed under the appropriate excitation-emission filters (Table 4.3) and the images produced were recorded and compared. The procedure was a modification of Lewitt and Pitt (2002).

Table 4.2: Sample specificity tests

Sample number	Anti-β tubulin antibody	FITC-labeled affinity purified to mouse IgG	Rhodamine-conjugates	Hoechst
Sample 1	-	+	+	+
Sample 2	+	-	+	-
Sample 3	+	+	-	+
Sample 4	+	+	+	-
Sample 5	-	-	-	+
Sample 6	-	-	+	-
Sample 7	+	+	-	-

Modified from Lewitt and Pitt (2002). The + sign indicates fluorophores being added to the samples and the – sign means elimination of the respective dyes.

4.4.3 Oocyte Preparation Methods

There were two methods of oocyte preparation. The first involved the application of different techniques of transferring oocytes prior to mounting step and the second was the procedure of preparing zona free and zona intact oocytes.

4.4.4 Poly-l-lysine Coated Coverslips Versus Microdroplets Technique

The poly-l-lysine coated coverslip preparation was described in Section 3.6.3.4. Oocytes were attached onto the coverslips prior to further treatments for ICC procedures. Another group of oocytes was treated by immersing them inside a series of microdroplets starting from fixation until mounting step. No poly-l-lysine coated coverslips was used for the second group. Oocytes were finally mounted onto glass slides for microscopic observation. The mounting medium utilised was glycerol and water in 1:4 ratio mixed with a small volume of anti-fading agent DABCO (1%). The images obtained from both techniques were studied and compared.

4.4.5 Zona Free Versus Zona Intact Oocytes

The zona free oocytes were prepared by dissolving the zona layer using acid's Tyrode as mentioned in Section 3.5.5.2. Another staining method utilised zona intact oocytes. They were subjected to the same ICC procedures and then attached onto the poly-l-lysine-coated cover slip. The images obtained were analysed and recorded.

4.4.6 Effect of Fixatives on Fluorescence Images of Oocytes

Two fixatives were used in this experiment. In one group, oocytes were fixed in formaldehyde (2%), while the other group was treated using cold methanol. They were then exposed to similar post-fixing treatments of ICC procedures (Tables 3.2a, b). Images produced from both of the fixation methods were compared.

4.5 EXPERIMENTAL DESIGN

There were four main parts of the experiments for optimisation of the ICC procedures. The first was on the optimal titre of the working dilutions, the second was the control

experiment for confirming reactivity of the antibody and fluorophores, the third was to compare the quality of the images taken from oocyte prepared using two different methods and the fourth was the effect of fixatives on oocyte structures. The quality of the images produced of the microtubules, the cortical granules and the nuclear materials were assessed.

4.6 MICROSCOPIC ANALYSIS

The results of the experiments rely primarily on microscopic observations of the cellular structures produced by the samples with definite fluorescence colour. The samples were analysed using the Nikon Ophtiphot UFX-DX microscope supplied with blue band-pass set filter BA420, green band-pass BA520 and red band-pass XF102-2 (Table 4.3). The images were captured using Nikon camera loaded with 400 ASA Kodak films, scanned and edited using Adobe Photo-editor. The fluorescent inverted microscope (Carl-Zeiss) with image analysis (Image Pro-Plus) was only available at the later stage of this project.

Table 4.3: Excitation-emission properties of the fluorescent filter sets used in the study

Microscope	Filter	Fluorophore	Emission Color	Excitation (nm)	Emission (nm)	Filter type
Nikon	BA420	Dapi/Hoechst (UV)	Blue	360-390	Not available	Long band-pass
	BA520	FITC	Green	450-490	Not available	Long band-pass
	XF102-2	TRITC	Red	560-615	645-720	Short band-pass
Carl-Zeiss	XF06	DAPI/Hoechst	Blue	365-415	450-515	Short band-pass
	XF100-2	FITC	Green	475-515	535-580	Short band-pass
	XF102-2	TRITC	Red	560-615	645-720	Short band-pass

Adapted from Molecular Probes (2008).

4.7 STATISTICAL ANALYSIS

Differences in the images of the oocyte structures between treatments were analysed statistically using descriptive analysis and Chi-square test at significance level $p< 0.05$. Analysis was executed using SPSS software version 12.0 and the observed and expected Chi-square values were compared in order to either accept or reject the null hypothesis (H_0: no difference between treatments). The structures of oocytes were evaluated by giving a code (1=present or 0=absent) for all the treatments afore-mentioned.

4.8 RESULTS

The descriptions of the results for the experiments were divided into four sections as below.

4.8.1 Optimisation of Titre Concentrations of The Antibody and The Fluorophores

The range of working dilution of E7 antibody varied between 1:10 to 1:20. The optimum dilution of FITC conjugates was 1:80 and a 10 µg/ml was the optimal concentration for both TRITC-LCA conjugates and Hoechst. Lastly, a working dilution consisting of 200 Unit per staining (Section 3.6.3.3.1) was adequate for Alexa-Fluor to stain F-actin. An apple green fluorescence image was the fluorescence colour of the microtubules, blue for the nuclear materials and red for both the cortical granules and the F-actin (Figure 4.1). The cortical granules and F-actin were stained separately on different samples because both structures must be observed using the same excitation-emission filter. Over expression of the fluorescence signals was occasionally observed in some samples (Figure 4.2) which resulted in diffused fluorescence staining and low

contrast especially during the trial periods. Some samples exhibited non-specificity when two colours such as the blue and the green fluorescence were emitted from the same structure (Figure 4.4). In some other samples, the nuclear materials displayed white fluorescence instead of blue while yellow fluorescence was observed for the microtubules instead of green. This could be related to high exposure time and inadequate filter system to reduce the amount of excitation energy that stroke the fluorescent probes. Similar problem occurred among the over-stained samples.

4.8.2 Control Samples

The summary of results is tabulated in Table 4.4. In general, deprivation of the primary antibody (E7) from sample 1 had resulted in no green fluorescence detected under BA520 filter. Red and blue fluorescences were observed under TRITC and UV filters, respectively. Only red fluorescence was observed for both samples 2 and 6 which were tagged with TRITC-LCA. Single dye staining for samples 5 and 7 showed blue in the former and green fluorescence in the later as expected. Even though the control samples had revealed the definite emission colour from a single dye staining, but overlapping of two to three fluorescence colours (cross-talk) were observed from some samples which underwent multiple staining using the same excitation filter (Figures 4.3 and 4.4) of Nikon Ophtiphot microscope. This microscope was supplied with long band-pass filters. There was none of such image produced from Carl Zeiss microscope which is equipped with short band-pass filters. The results on non-specificity (cross-talk) test are given in Table 4.5 and Figure 4.3. Of the total observations (n=101), only 30 images (29.7%) displayed cross-talked fluorescence colours. Further analysis revealed that the incidence of cross-talk was more prominent under the UV filter, through which 61.5% of the images had produced both blue

(nuclear materials) and green (microtubules) fluorescence colours simultaneously as compared with FITC and TRITC filters, which were 15.0% and 0.0% of cross-talks, respectively ($p<0.001$).

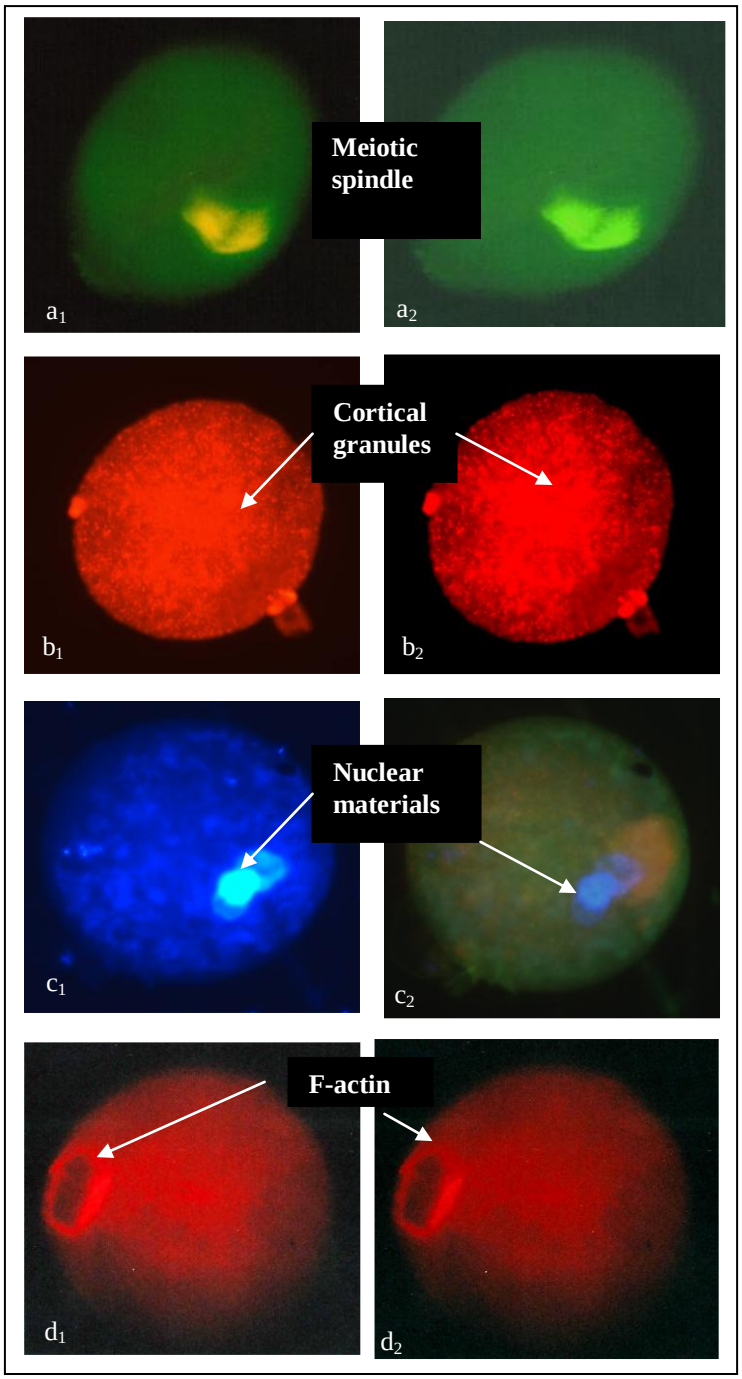

Figure 4.1: Fluorescence images of the mouse oocytes showing strong signals of FITC for meiotic spindle (a_1), TRITC for cortical granules (b_1), Hoechst for nuclear materials (c_1), Alexa-Fluor for F-actin (d_1) and the corresponding images (a_2), (b_2), (c_2) and (d_2) of the respective structures edited by using photo-editor software. (c_2) is the composite image of the three dyes. (400x)

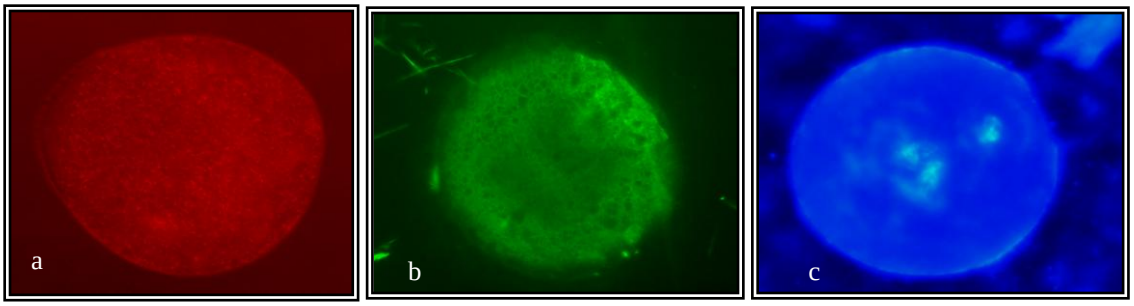

Figure 4.2: Fluorescence images resulted from improper staining procedure. High background of TRITC (a), FITC (b) and Hoechst (c) could have been resulted from inadequate washing or high concentrations of the dyes used. (400x)

Table 4.4: Summary of results from the control samples

Sample number	Anti-β tubulin antibody	FITC-conjugates	Rhodamine-conjugates	Hoechst	Filter 1 (450-490 nm	Filter 2 (560-590 nm	Filter 3 (360-390 nm)
Sample 1	-	+	+	+	-	Red	Blue
Sample 2	+	-	+	-	-	Red	-
Sample 3	+	+	-	+	Green	-	Blue
Sample 4	+	+	+	-	Green	Red	-
Sample 5	-	-	-	+	-	-	Blue
Sample 6	-	-	+	-	-	Red	-
Sample 7	+	+	-	-	Green	-	-

+ addition of fluorophores to samples
- no fluorophores being added/absence of fluorescence signal

Table 4.5: Cross tabulation between type of filters and cross-talk in the fluorescence images of the mouse oocyte

Filter	Fluorophore	Total count	Number of cross-talk	Percentage (%)
BA 420	Hoechst	39	24	61.5
BA 520	FITC	40	6	15.0
XF102-2	TRITC	22	0	0.0
Total (n)	3	101	30	29

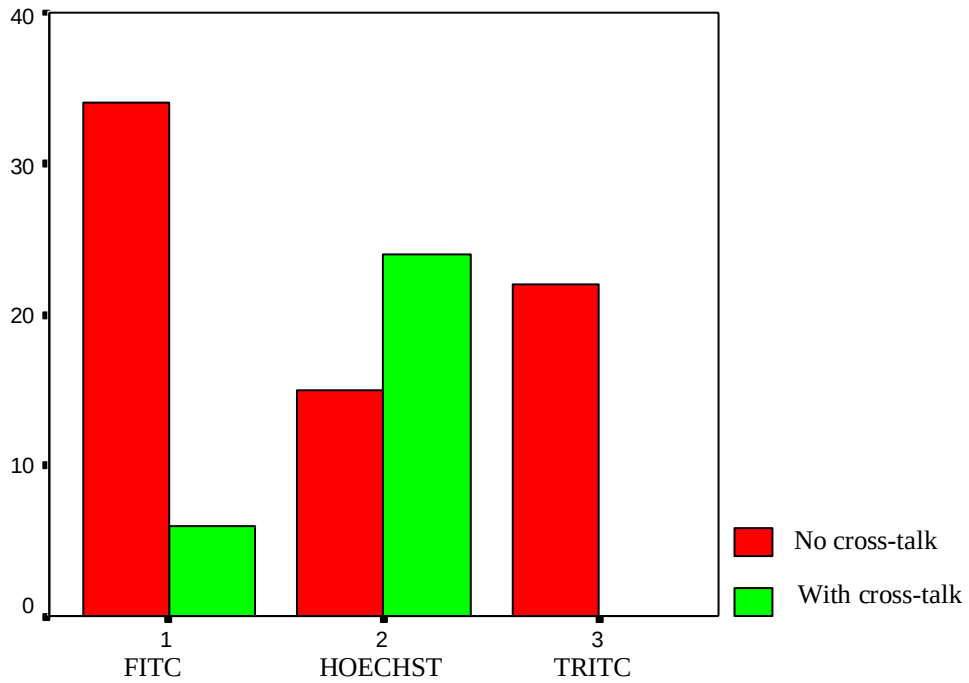

Figure 4.3: The number of images with cross-talk between the filter sets.

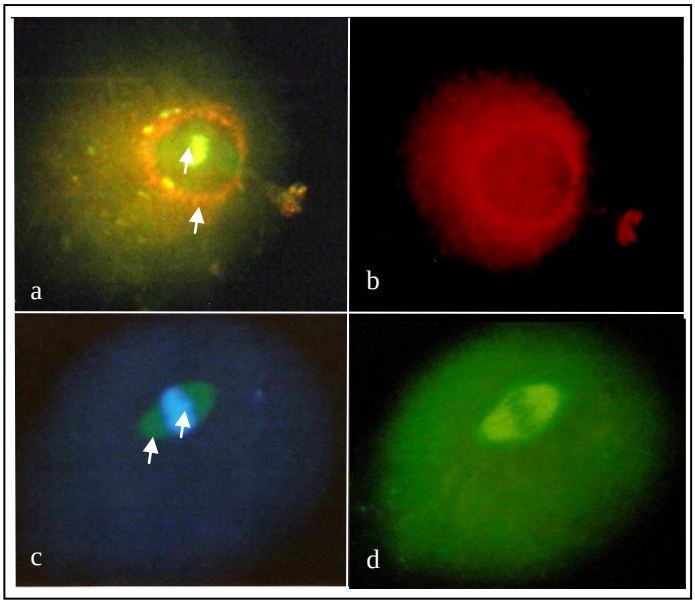

Figure 4.4: Cross-talk of fluorescence signals (arrows) in images (a) and (c). Green and red (a) appeared under BA520 filter while green and blue (c) under UV range. The corresponding images (b) and (d) were taken under TRITC and FITC excitation, respectively. All Images were recorded using Nikon Opthiphot fluorescent microscope. (400x)

4.8.3 Effect of Sample Preparation on Fluorescence Images of Mouse Oocytes

The fluorescence images of the mouse oocytes obtained from different sample preparations were compared and discussed in the following sections.

4.8.3.1 Poly-l-lysine coated versus microdroplets preparation

The aims of the experiment were of two folds. The first was to determine the number of cell recovered and the second was to compare the quality of images produced. The number of oocytes adhered onto poly-l-lysine coated coverslips was relatively poor and many were eventually detached and loss during the staining procedures especially during repetitive washing steps. The mean value of recovery rate for oocytes prepared onto poly-l-lysine coated coverslip was significantly lower than that of the microdroplet

method, which were 30.4±8.9% and 73.8±6.15%, respectively ($p<0.01$). Analysis on the images produced from both methods was conducted qualitatively by referring to the sharpness and clarity of the images of the cellular structures. Comparable results were found between the methods.

4.8.3.2 Zona free versus zona intact oocytes

Cellular structures for both zona free and zona intact oocytes which included the microtubules, nuclear materials and cortical granules were quantitatively and qualitatively analysed. The results are tabulated in Table 4.6. Statistical analysis showed that of the total 129 zona intact oocytes stained with FITC conjugates only 81 (62.8%) had revealed the clear image of meiotic spindles. In contrast, a significantly lower percentage was obtained among the zona free which were 96 samples out of 250 or 38.4% ($p<0.001$). On the other hand, comparable images were obtained for the nuclear materials in both treatments which were approximately 83.0% and 86.8%, respectively, were detected from both the zona intact and zona free groups of oocytes ($p>0.05$). Although higher percentage was observed for the cortical granules appearance among the zona intact than the zona free, which were 90.0% and 86.4%, respectively, however, the analysis showed that the difference was not significantly affected by the zona layer ($p>0.05$) .

Table 4.6: Comparative analysis on oocyte structures between zona intact and zona free oocytes

Oocyte	Meiotic spindle (%)	Nuclear materials (%)	Cortical granules (%)
Zona intact	62.8^{a} n=129	83.0^{a} n=205	90.9^{a} n=55
Zona free	38.4^{b} n=250	86.8^{a} n=312	86.4^{a} n=140

Supercripts ^{a,b} in a column differ significantly at $p<0.05$.
n=Total number of oocytes

Comparative fluorescence images of the oocyte morphology from both groups are shown in Figures 4.5 and 4.6, respectively. The TRITC-LCA fluorophore stained the cortical granules to produce red fluorescence. Meanwhile tubulin and the nuclear materials were frequently observed only in some of the oocytes. Non-specificity of fluorescence reactivity was observed in both of the images shown in Figures 4.5b, c, which were recorded under FITC and UV excitation filters, respectively.

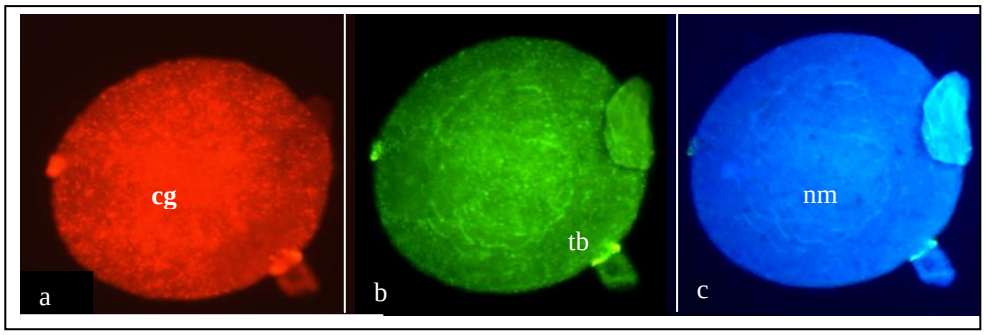

Figure 4.5: Zona-free oocyte stained with TRITC-LCA (a), FITC-IgG (b) and Hoechst (c). cc-cumulus cell, cg- cortical granule, nm-nuclear materials, tb-tubulin. (400x)

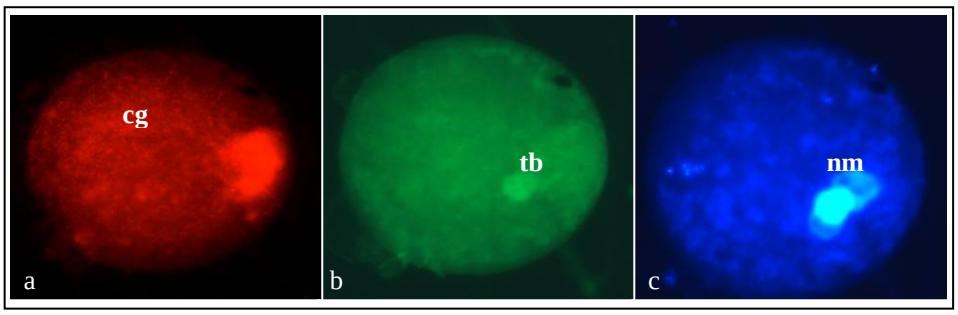

Figure 4.6: Zona-intact oocyte stained with TRITC-LCA (a), FITC-IgG (b) and Hoechst (c). cg-cortical granule, nm-nuclear materials, tb-tubulin. (400x)

As shown in Figure 4.6, strong fluorescence signals were detected for all the samples prepared especially in Figure 4.6c, which was tagged with Hoechst. This indicated that

zona posed insignificant barrier for penetration of fluorophores to label the respective structures of mouse oocytes especially the nuclear materials.

4.8.4 Effect of Fixatives on Fluorescence Images of Oocytes

The integrity of oocytes fixed by methanol and formaldehyde was qualitatively and quantitatively compared and summarised in Table 4.7. The results showed that the images for the meiotic spindles, but not the nuclear materials and the cortical granules were significantly affected by the type of fixative used ($p<0.001$). Analysis showed that the number of oocytes with abnormal or patchy nuclear materials were considerably low for both groups. Of the total of 275 oocytes fixed in formaldehyde (2%) only 32 (11.6%) displayed derangements of the nuclear materials as compared with 32 (13.2%) out of 242 being fixed in methanol ($p>0.05$). Most of the oocytes in both groups clearly displayed the nuclear materials (83.3% and 86.0%, respectively) and the cortical granules (86.4% and 90.0%, respectively). On the contrary, a significantly higher percentage of oocyte displaying meiotic spindles were those being fixed in methanol rather than in formaldehyde which were 64.7% and 34.5%, respectively ($p<0.001$).

Table 4.7: The percentages of fluorescence images revealed by oocytes fixed in formaldehyde and methanol

Fixative	Meiotic spindle (%)	Cortical granule (%)	Normal nuclear materials (%)	Deranged nuclear materials (%)
Formaldehyde (2%)	34.5[a] n=226	86.4 n= 125	83.3 n=275	11.6 n=275
Methanol (absolute)	64.7[b] n=153	90.0 n=70	86.0 n=242	13.2 n=242

Superscripts[a,b] in a column differ significantly at $p<0.001$
n= total number of oocytes

4.9 DISCUSSION

In this experiment, the nature of results primarily obtained using ICC procedures were in the form of fluorescence micrographs, in which its quality depended greatly on a multiple of factors among which were the procedures employed, the reagents used, the equipment and most importantly the skill of personnel involved. Immunocytochemistry has been established for the past two decades and it is no longer a breakthrough technology in many laboratories which are actively involved in advanced studies of cells. Nevertheless, establishing the method for the first time in our own laboratory requires preliminary testing in various aspects for the purpose of validating the efficiency and efficacy of the methods employed that would eventually affect the final results. This is crucially important in determining the compatibility of the system or instruments involved with the procedures employed for detecting the specific cellular structures under study. Once established, the efficiency and efficacy of the tested system can be relied upon for producing quality fluoresecence images with minimal external interferences.

The fluorescence images obtained clearly showed that the developed ICC protocols have been successfully established and suitable for studying oocyte structures in our laboratory. However, some modifications and improvements on the procedures are to be made. These included upgrading of instruments with advanced imaging analysis that is highly recommended from which detail microscopic analyses of the images could be conducted. It is believed that more significant information on the cellular structures under study could be extracted with advanced devices of microscopy technique.

The aim of the first experiment was to determine the optimum titre concentrations for the antibody and the fluorophores, which stained the respective

oocyte structures, namely the cytoskeletal, cortical granules and the nuclear materials. The titre concentrations used were within the range as recommended by the respective manufacturers and other laboratories. For examples, the concentration for E7 was in the range of 1:10 to 1:20, which resembled an earlier report (Sutovsky *et al.,* 1996). Similarly, the range of Hoechst for DNA staining was 10 µg/ml, which was equivalent to the concentration reported by others (Meng and Wolf, 1997; Hiroyuki *et al.,* 2002). Variation in the concentrations depended greatly upon the life span of the reagents as well as proper handling and storing. Varied concentrations used were due to its reduced sensitivity resulted from technical problems and mishandling, for instance, the antibody was unintentionally exposed to room temperature over a long period of time while being delivered to the laboratory and power failures. This indicated that acquaintment of skill and knowledge on the safe and proper keeping of the reagents is crucial among the personnel involved. In addition, a research-friendly environment should be inculcated for all laboratory members especially when sharing of resources is highly practised. Most reagents used in ICC are proteins in nature, and must be kept frozen. Other factors concerned with proper handling and storing were frequent freezing and thawing of the aliquots which had contributed to its reduced sensitivity and immediate bleaching when exposed to light. Thus, it is recommended to aliquot working solutions in small volumes and stock solution is kept frozen at all times.

It is also advisable to conduct the procedures inside a dark room or with minimum light, especially during the microscopic analysis. Detecting the optimum concentration of each stain is beneficial not only for producing quality images but also cost effective. Inaccuracy resulting from either over-staining (Figure 4.2) or diluted fluorophores would jeopardise the final results. For example, additional steps and longer hours of rinsing were necessary to remove noisy background from over-stained

samples through which the contrast between the structures and background can be improved.

The quality of fluorescence images would depend primarily not only on the sample and staining procedures but also suitability or compatibility of the fluorophores with the microscope used especially the properties of the filter sets. This was specifically highlighted by the results which showed that the short band-pass filters emitted only single (specific) fluorescence instead of two overlapping colours from the long band-pass filters. The two-colour bearing images (cross-talk) indicated non-specificity of the emitted light in which two fluorophores were emitted within the same emission spectra. These were found to be higher from the UV filter than the green filter at higher excitation wavelength, which were 61.5% and 15%, respectively. The fact that energy of photon is inversely proportional to the wavelength (WL) showed that UV exposure with greater energy resulted in excitation of more than one fluorescence signals and simultaneously emitted the lost energy in the forms of multiple fluorescence colours. The significant differences ($p<0.0001$) in the specificity of filter types was proven by the Chi-square analysis. Samples stained with single fluorophore were not affected by the filter types and the discrepancies attributed by cross-talk could be alleviated and ascertained through the images obtained from the control samples. This is inevitably needed and highly recommended if the existing system is to be utilised for future research. In other words, multiple staining may be affected unless new filter sets are to be installed; hence, upgrading the present system and instrumentation is recommended. The said problem was not observed in the Carl Zeiss system, which was obtained at the very late stage of the study. In addition to having short band-pass filter sets, it is equipped with advanced image analysis that is capable of highlighting the cell structures even though the signal was very weak (low detection of fluorescence colour

due to photo-bleaching) as well as adjusting the strong fluorescence signal from the samples using programmable exposure hours. The results implied that suitability and compatibility between the microscope, fluorophores and the filter sets are crucial for establishing ICC procedures in a laboratory. A very good knowledge and acquaintance on the operational aspects of the fluorescent microscope would be advantageous for conducting the ICC protocols. This would eventually be beneficial for advanced study of cellular structures and imaging.

The results of this study also revealed that there was no difference in terms of images obtained between the poly-l-lysine coated and the microdroplets sample preparations. However, the mean percentage of the number of oocytes recovered was greater from microdroplets preparation than poly-l-lysine coated, which were 73.8±6.15% and 30.4±8.9%, respectively ($p<0.01$). This indicated that poly-l-lysine coated procedure requires prudence preparation due to several factors among which oocytes are round and not flat, hence, it requires adequate time and the right concentration of the reagent for the cell to be attached firmly. Moreover, coated slides picked up the secondary fluorescence tag somehow and were noisy (Figure 4.2). Other factors found to be important were to use of extremely cleaned coverslips or glass slides and protein-free (BSA or serum free) culture medium (Simerly and Schatten, 1993) for preparing oocytes at constant temperature 37^{o}C. It was also noted that small volume of microdroplets (50 μl) of the reagents used in the protocols gave better cell attachment. Cell detachment could be attributed to cell floating or cell lysis and this was severely affected during repetitive washing and staining. It could also be affected by foreign elements such as dusts or microorganisms attached onto the slides. Thus, it is recommended that most bench works should be conducted inside an enclosed dust free environment. Any negligence could compromise the cell count. On the contrary,

microdroplets preparation was proven to improve the number of oocytes recovered at the end of the staining procedures. In addition, oocytes were mounted directly into a small volume (30 μl) of medium on a glass slide, which resulted in most of them being grouped near the centre of the coverslip and easily detected during microscopic observation. This would minimise searching efforts and reduce the time span of light exposure on sample, hence, less effect on fluorescence bleaching. Also, it would be very cost effective since only small volume was needed for preparing a large group of oocytes. Coverslip size and type did play some roles, in which for this study the #1 instead of #1.5 coverslip could reduce the number of smashed cells, however, chambered coverslip slides are highly recommended (Anderson, 2008). Tae Do (2002) had successfully immunolocalised the microtubules and chromatin in ICSI bovine oocytes using four-well dish.

Many ICC procedures utilised zona free oocytes for fluorescence microscopy technique (Schatten *et al*., 1985; Sutovsky *et al.*, 1997). Simerly and Schatten (1993) stated that permeabilisation is not complete unless zona free oocytes are used. In addition, zona removal is necessary to attach oocyte onto coverslip prior to subsequent ICC protocols because the oocytes would be flattened off and localised at specific spot. This would ease the process of fixing and antibody penetration as well as image processing during microscopic analysis. Little information was obtained on ICC application with zona intact mouse oocytes. It was reported that detergent such as TritonX-100 could disrupt membrane polarity and also caused membrane extraction. This would help antibody penetration into the samples (Jackson laboratory, 2008). The results of this study revealed that the percentages of each the nuclear materials and the cortical granules images obtained from both zona free and zona intact groups were insignificantly different ($p>0.05$) but not for the microtubule images. The appearance

of meiotic spindles was significantly higher in the zona intact than the zona free, which were 62.8% and 38.4%, respectively ($p<0.0001$). This was probably due to the fact that microtubules were more stable and protected inside the zona intact than the zona free oocyte. The preliminary preparation of the oocytes for ICC application such as zona dissolution using acid Tyrode's probably had resulted in instability of the microtubular structures. Also, longer exposure to acid Tyrode's could have dissolved the polar body and its contents too; hence, disrupting its structural linkage with the oocyte. The results indicated that ICC application on mouse oocyte is feasible with or without zona for studying nuclear materials and cortical granules but not meiotic spindle. The use of microtubule stabilising buffer (Schatten *et al*., 1985) prior to fixation and during permeabilisation steps is highly recommended. Also, oocytes which were preliminary denuded with acid Tyrode's should be reincubated inside the culture medium prior to fixation. This step helped to increase antibody penetration and reduced non-specific antibody binding (Simerly and Schatten, 1993). It is also reported that the use of sodium azide in permeabilising medium would help to prevent internalisation of the antibody–antigen complex after the antibody bind to the receptors (Jackson laboratory, 2008). Zona intact oocytes were best prepared inside microdroplets or four-well dish (Tae Do, 2002) to prevent cell loss.

The final part of this experiment was to compare the effect of fixatives on the fluorescence images of the oocytes. Comparative analysis revealed that meiotic spindles were not only affected by the zona layer but also the fixative used. This finding validated product description that meiotic spindles labeled with E7 antibody would be best detected in methanol-fixed oocytes (Hybridoma Bank-The E7 supplier). This is also in lined with previous reports, that formaldehyde may cause extensive cytoskeletal lysis and damage on mouse oocytes when aqueous solutions are applied

during subsequent rinsing steps (Simerly and Scahttten, 1993). However, both the nuclear materials and the cortical granules were not affected by the types of fixative used. Some other factors to be considered are pH, osmolarity, temperature and time of incubation. In summary, qualitative and quantitative assessments of improvised ICC method include the following:

a) *It provided satisfactory recovery rate and reduced cell loss* The number of cell recovered during ICC staining procedures was satisfactory and the amount of cell loss was reduced.

b) *It was economical and cost effective* Low volume of reagents (fixative and dyes) was adequate to be used per treatment. As little as 50 to100 µl volumes of fixatives, stains and other reagents were adequate in the staining steps. A large cohort of oocytes or embryos could be stained simultaneously per microdroplet instead of separating them into several batches. Some procedures could be omitted such as the applications of poly-l-lysine coated cover slips for cell adherence and the dissolution of zonae, hence, eliminating the use of poly-l lysine and acid Tyrode's solution; less stress imposed on oocytes. The procedure required less expenditure on coverslips and glass slides. One or two slides were adequately enough to mount the entire groups of cells. Moreover, the 4- and 6-wells dishes could be recycled.

c) *It was flexible and high reproducibility* Procedures could be easily repeated. The whole procedures were less destructive on oocyte structures, especially among the zona intact oocytes. The application of acid Tyrode's might destabilise oocyte microtubules, thus, improved medium for permeabilisation of cells as well as stabilisation of microtubules is highly recommended.

d) *Time saving* The entire procedures could be completed relatively quickly in a day of working period as compared to two consecutive days needed for the conventional method. Also, cells could be easily located and searched under the microscope. The cells were placed on a glass slide with little drop of mounting medium. Distribution of cells wass limited within the cover slip, visible and easily located.

e) *High contrast* The non-poly-l-lysine coated coverslips produced clean background. Thus, clearer image could be obtained due to minimal noisy background. Also, little solvents used might reduce autofluorescence. Various intracellular structures especially the cytoskeleton was easily detected in both zona free and zona intact oocyte by using control samples as the structure of reference.

4.10 CONCLUSIONS

In conclusions, the ICC technique which followed Simerly and Schatten (1985, 1993) to study oocyte morphology had been successfully established in our laboratory with some modifications. Despite conventional imaging technology used, improvised ICC method perfectly improved cell recovery by using zona intact oocyte prepared in microdroplets for staining procedures. The study is feasible with reduced staining steps, efficient and cost effective. Most importantly the efficiency of the technique had been validated through several preliminary tests, which had eventually resulted in reduced number of cell loss, easy detection of cell location, time saving and comparable end results. Precision and resolution for cell-imaging depended upon the properties of optical devices of the fluorescent microscope used. The suitability and compatibility of the microscope set up with the fluorophores should be identified prior to establishing the

procedure. Hence, the established technique pays dividends to the field of study, which continues to expand concomitantly with new technology available in optical devices and softwares in cell-imaging. In addition, more diverse range of fluorophores with better sensitivity and produce faster effects could be used. This would make the study of cell especially mammalian oocytes using ICC technique to be a more fascinating research work to explore.

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