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Aurora A localization in mouse oocytes is coupled to meiotic spindle integrity: Effects of demecolcine-induced microtubule destabilization

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Abstract

The family of serine/threonine proteins known as Aurora kinases have been shown to play an important role in cellular proliferation as key regulators of cell cycle progression in mitotic cells. During meiosis, Aurora A is required for proper microtubule organization, establishing and sustaining spindle integrity, and proper chromatid alignment. This study investigated the effects of demecolcine, a microtubule destabilizing drug, on spindle localization of Aurora A in CF-1 mouse oocytes prior to, during, and following parthenogenic activation. This series of experiments tested the hypothesis that demecolcine enduced enuculation may affect critical cytoplasmic determinants required for proper embryo development. Marked differences were observed between controls and demecolcine treated oocytes with respect to the disregulated spatial distribution of Aurora A. In particular, dissimilarites in activation were observed after a period of only 20 minutes. Demecolcine treated oocytes contained spindles that were slow to rotate and displayed continued expression of spindle pole localized Aurora A. These results suggest that demecolcine disrupts cytoplasmic factors, such as Aurora A, in a time dependent manner.

Acknowledgements

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Chapter 1: Introduction

Methods for conventional somatic cell nuclear transfer involve the process of inserting the genome of a donor into an enucleated oocyte. This insertion is performed either via electroporation or microinjection; two procedures which are not only time consuming but also quite invasive and may contribute to the reported limited success of somatic cloning thus far (Ibáñez et. al., 2003). These approaches may also deplete the oocyte cytoplasm of important factors that are required to complete subsequent development. An alternative to mechanical enucleation which has been reported is a method of chemical enucleation that incorporates a microtubule destabilizing drug, demecolcine. When activated oocytes are treated with demecolcine, they continue to undergo normal cytoplasmic division (cytokinesis) and thereby complete formation of the second polar body. In contrast, nuclear division (karyokinesis) is disrupted causing all of the nuclear DNA to be extruded into the second polar body during the completion of meiosis II. This procedure has been demonstrated to potentially increase the developmental competence of cloned embryos (Ibáñez et. al., 2003).

A significant problem associated with the conventional methods is, that following oocyte reconstruction, the nuclear transfer embryo demonstrates blocked cell cycle progression. The family of Aurora kinases are known to be key cell cycle regulators, and Aurora A in particular is responsible for the regulation of microtubule-based spindle assembly (Yao et. al., 2004). Increased microtubule organization is achieved through the phosphorylation of the centrosome protein TACC by the protein kinase, Aurora A (Brittle and Ohkura, 2005). Studies on microtubule organizing centers have reported that the

presence of Aurora A is required for proper assembly (Tsai and Zheng, 2005) while over expression of Aurora A has been linked to breast, colorectal, and gastric cancers (Andrews, 2005). Improper chromatid separation can result in the formation of tumors due to decreased gene stability and anuploidy (Bolanos-Garcia, 2005). By observing the localization patterns of Aurora A in activated oocytes in response to demecolcine-induced altered spindle integrity, conclusions can be made with respect to the role of this key cell cycle regulator in subsequent development of cloned embryos.

Chapter 2: Background

Mouth pipetting

The standard method for moving oocytes is through a technique known as mouth pipetting. Using an apparatus including a drawn-out glass Pasteur pipet, rubber tubing, a filter, and a mouth piece microscopic items can be transferred from one location to another. Due to the small size of the oocytes (85 um) glass Pasteur pipets must be pulled to decrease their diameter. Pulling pipets is a process by which a Pasteur pipet tip is introduced to a flame and, once heated, is pulled to a smaller diameter more suitable for the experimental model.

Oocyte Manipulation

Before attempting to move actual oocytes, it is essential to become acquainted with the tools and methods that will be required. In order to study the effects of demecolcine using a mouse model, oocytes must be collected from the donor, treated with the appropriate experimental conditions, and then stained to aid in the visualization the localization of structures and proteins. This process requires the ability to accurately separate and transfer oocytes from one medium to another. Familiarity with the stereomicroscope while using pulled Pasteur pipets for mouth pipetting can be aided through the use of blue beads. While these beads are denser than actual oocytes they are a good representation of the small size. Once comfortable with transferring blue beads, continued practice with fixed oocytes can aid not only in the ability to relocate them, but also to identify the difference between fragmented, lysed, and healthy oocytes.

Mouse Handling and Peritoneal Injections

Both hormones used to induce superovulation in female mice require the ability to properly administer peritoneal injections. To be able to accurately inject the necessary hormones, proper handling that is both safe for the mouse and handler needs to be accomplished. Mice can be restrained by holding the scruff close to the back of the head with the thumb and index finger while holding the tail down with the pinky finger. A 1/2" 27 gauge needle on a 1ml syringe should be used to inject the proper hormone interperitoneally taking care to avoid the bladder.

Collection procedure

The mouse donor must be humanely killed prior to harvesting the superovulated oocytes. For this project mice were euthanized using carbon dioxide. The animals were prepped with 70% ethanol prior to collection to reduce the possibility of contamination by mouse hair. The skin over the abdomen is cut with surgical scissors and then pulled back to reveal the peritoneum. Using forceps and smaller surgical scissors the peritoneum is then cut to expose the viscera. Intestines should be moved to expose the uterus which needs to be separated from the mesometrium. The uterus should be traced back to the ovary which can be held with watchmakers forceps. Using the small surgical scissors the oviduct should be removed and placed in oocyte culture medium (FHM). Using the watchmakers forceps, a syringe, and a microscope the ampulla of the oviduct should be located and pierced to release the cumulus mass containing the oocytes.

Chapter 3: Materials and Methods

Collection of oocytes

Female CF-1 mice (Charles River Laboratories, MA) were superovulated using Pregnant Mare Serum Gonadatropin (PMS, 5IU) followed 48 hours later by human Chorionic Gonadatropin (hCG, 5IU). Oocytes arrested at metaphase of meosis II (MII) were collected 16 hours post hCG in FHM. Oocytes were denuded of adherent cumulus cells by brief incubation in the presence of hyaluronidase at 37°C, then washed, and fragmented oocytes were discarded. Oocytes were randomly pooled and assigned to treatment groups.

Experimental Design

Oocytes were activated by continuous exposure to strontium chloride ($SrCl_2$, 10mM) for 20 minutes, 45 minutes, 1.5 hours, 2 hours, and 4 hours. Three groups were employed: a control group (MII-stage oocytes, n=37), a treatment control (activated oocytes, n=171), and a treatment group (oocytes activated in the presence of 0.4ug/ml demecolcine, n=162). Additionally, an experiment was conducted to assess the reversibility of demecolcine treatment. This consisted of oocytes (n=30) activated with $SrCl_2$ and 0.4ug/ml demecolcine for 45 minutes and were then moved to demecolcine free activation medium for an additional 45 minutes.

Fixation, Staining, and Immunofluorescence Microscopy

All oocytes were fixed in 1% paraformaldehyde (PFA) with 0.15% Triton X-100 in PBS for 30 minutes at 37°C either immediately after collection (MII) or at specific time points post-activation. Oocytes were stained using antibodies specific to the desired cellular proteins. Aurora A was stained using a primary rabbit anti-Aurora A polyclonal antibody (Zymed) followed by a secondary goat anti-rabbit IgG fragment labeled with alexa fluor 594 (5ug/ml, red, Molecular Probes). Microtubules were probed with a primary mixture of mouse anti-α-tubulin and anti-β-tubulin monoclonal antibodies (1:1000 dilution, Sigma) followed by a secondary goat anti-mouse antibody labeled with alexa fluor 488 (5ug/ml, green, Molecular Probes). Chromatin was stained using Hoechst 33258 (10ug/ml, blue). Stained oocytes were transferred to slides with mounting solution (50% glycerol, 50% PBS, 25mg/ml sodium azide), carefully covered with a cover slip, and sealed with clear nailpolish. Imaging was performed using a Zeiss Axiovert 200M inverted microscope with a Roper CoolSnapFx CCD camera. MetaMorph software was used to collect images in real time.

Antibody optimization

Prior to beginning the experimentation, a decision regarding antibody selection needed to be made. When staining for Aurora A there are two options: either a monoclonal or a polyclonal antibody. The benefit of the monoclonal antibody is its specificity, however if the desired target is rare in quantity, sufficient primary binding may limit visualization. Alternatively, the second type of antibody, the polyclonal, will react with multiple epitopes specific to the protein of interest, thereby increasing

visualization. In general, non-specific binding is reduced when using a monoclonal versus a polyclonal antibody and this feature needs to be taken into account. In order to determine which type of antibody to use two optimization experiments were performed.

MII oocytes (total=128) were used to compare and determine the optimal concentration of a monoclonal and polyclonal Aurora A antibody with a minimum of 11 oocytes in each treatment group. Oocytes were introduced to varying concentrations of each antibody (1:100, 1:500, or 1:1000) or one of two negative control groups (primary antibody only, secondary antibody only) where block was used in place of the eliminated antibody. After imaging the oocytes, it was concluded that the monoclonal antibody worked best at a concentration of 1:500, while the polyclonal antibody was clearest at a concentration of 1:100. Based on the knowledge of the localization of Aurora A to the spindle poles, it was determined that the polyclonal antibody would be most appropriate for this experiment due to its compatability for co-localizatioin of tubulin staining using a monoclonal antibody. The staining protocols used for the monoclonal and polyclonal antibodies can be found in Appendix A and B respectively.

Chapter 4: Results

As shown in previous reports, Aurora A was localized to the spindle poles in MII-stage oocytes (Figure 1). Following activation, the localization of Aurora A was diminished during anaphase of meiosis II (Figure 2 A&B, 3 A&B) and then relocalized at the spindle poles as the oocytes progressed to telophase of meiosis II (Figure 4 A&B). Upon reaching interphase (I), the Aurora A localization was once again reduced (Figure 5 A&B). The demecolcine treatment groups (Figures 2 C&D, 3 C&D, 4 C&D, 5 C&D) displayed not only disrupted microtubule organization and decreased spindle integrity but also altered Aurora A localization. In these treatment groups, the activation process appeared to be delayed as indicated by lack of spindle rotation and the continued localization of Aurora A after 20 minutes. In the later time points, lack of second polar body extrusion was observed in the demecolcine treated oocytes. In the 1.5 hour recovery experiment (Figure 6), spindle integrity and rotation, and Aurora A localization did not revert to a normal phenotype within the observed time frame.

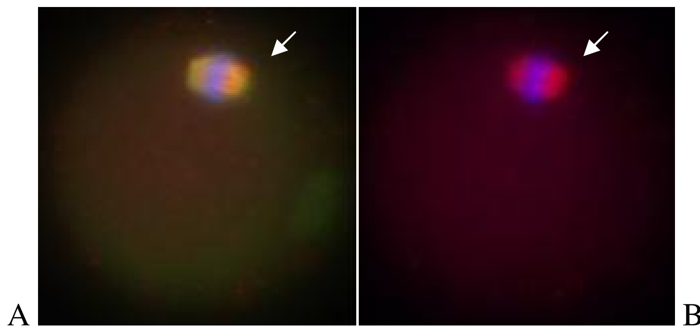

Figure 1: MII-stage oocyte stained for Aurora A (red), tubulin (green), and chromatin (blue) in the left image (A) and Aurora A and chromatin in the right image (B).

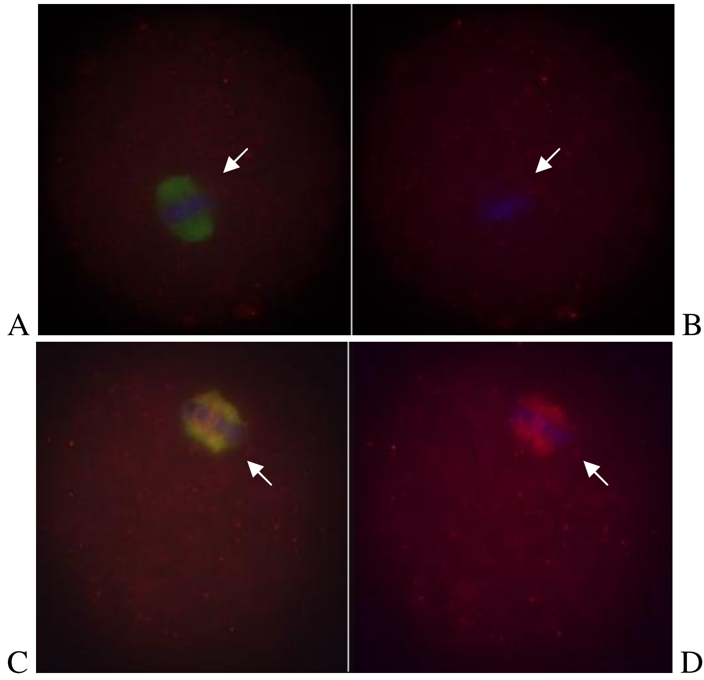

Figure 2: 20 minute activated control (A&B) and demecolcine treated (C&D) oocytes. For each oocyte, Aurora A (red), tubulin (green), and chromatin (blue) staining is shown in the left image (A, C) and Aurora A and chromatin staining is shown in the right image (B,D).

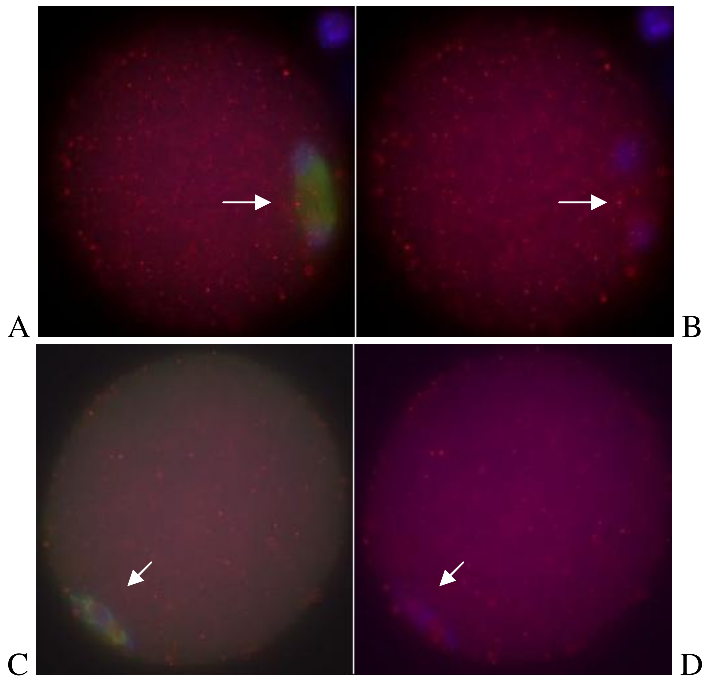

Figure 3: 45 minute activation control (A&B) and demecolcine treated (C&D) oocytes. For each oocyte, Aurora A (red), tubulin (green), and chromatin (blue) staining is shown in the left image (A,C) and Aurora A and chromatin staining is shown in the right image (B,D).

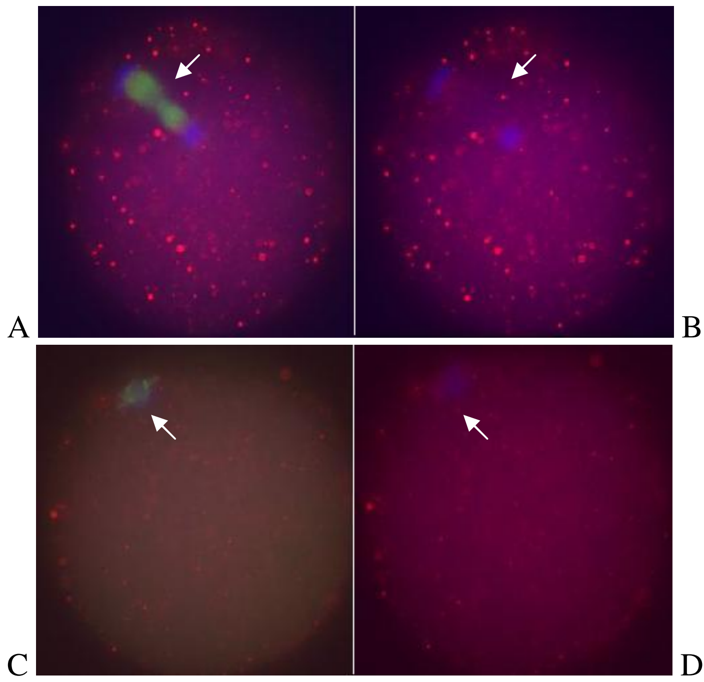

Figure 4: 2 hour activation control (A&B) and demecolcine treated (C&D) oocytes. For each oocyte, Aurora A (red), tubulin (green), and chromatin (blue) staining is shown in the left image (A,C) and Aurora A and chromatin staining is shown in the right image (B,D).

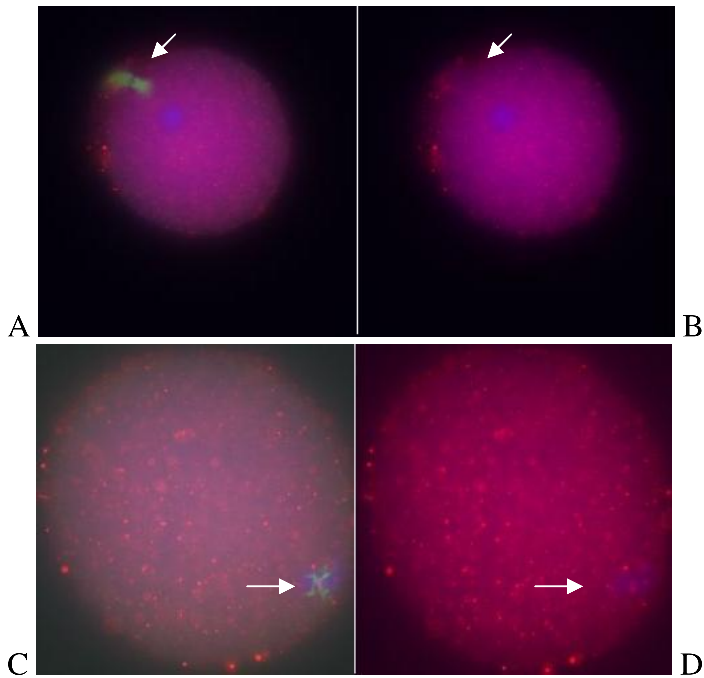

Figure 5: 4 hour activation control (A&B) and demecolcine treated (C&D) oocytes. For each oocyte, Aurora A (red), tubulin (green), and chromatin (blue) staining is shown in the left image (A,C) and Aurora A and chromatin staining is shown in the right image (B,D)

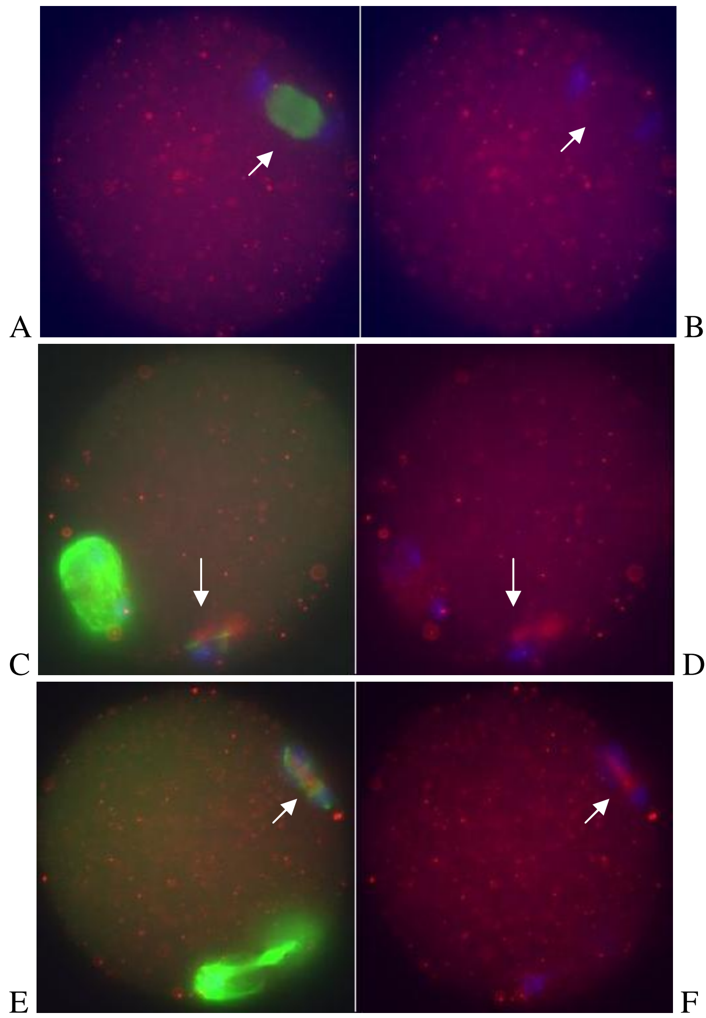

Figure 6: 1.5 hour activation control (A&B), demecolcine treated (C&D), and recovery (E&F) oocytes. For each oocyte, Aurora A (red), tubulin (green), and chromatin (blue) staining is shown in the left image (A,C,E) and Aurora A and chromatin staining is shown in the right image (B,D,F)

Chapter 5: Discussion

Normal meiosis II progression is indicated by chromosomes lining up at the metaphase plate, followed by 90° spindle rotation, chromatid separation and segregation to the spindle poles, and the extrusion of the second polar body (Ibáñez et. al., 2003). The introduction of demecolcine into the activation medium results in altered spindle integrity due to the drugs microtubule depolymerizing properties. A lack of spindle rotation in demecolcine treated oocytes has previously been reported (Ibáñez et. al., 2003). In this study, a similar effect was observed using strontium chloride as the activator. Another similarity observed in these two studies is the lack of complete second polar body extrusion in the majority of demecolcine treated oocytes. Here, like in the Ibáñez report (2003), the presence of demecolcine induces depolymerization of microtubules causing spindle collapse. Similarities were also observed when compared to a study on Aurora A localization during fertilization and early embryo development (Yao et. al., 2004). In both cases, Aurora A was localized to the spindle poles in MII-stage mouse oocytes and then diffuse after the extrusion of the second polar body following 2-4 hours of activation.

In general, the demecolcine treated oocytes displayed delayed activation as indicated by deferred spindle rotation and residual Aurora A localization which differed from the patterns observed in control oocytes. Additionally, there was a lack of observed second polar body extrusion in the treatment groups. In the demecolcine recovery experiment, normal spindle configuration and Aurora A localization was not achieved in

the observed time frame. This indicates a need for more information regarding the reversibility of demecolcine effects.

Based on these data, further studies should evaluate the effect of demecolcine on the timing and spatial expression of other key cell cycle regulators in activated mammalian oocytes. Such information may lead to a better understanding of the cellular mechanisms that support successful somatic cell cloning.

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Appendix A: Monoclonal Aurora A Staining Protocol

Triple staining of mouse oocytes/embryos (Aurora A monoclonal, f-actin, chromatin)

Fixed oocytes/embryos kept at 4 degrees C in block solution at least overnight before starting staining procedure.

Step	Time	Temp
1. Primary Ab: mouse anti Aurora A	60 min	37°C
2. Wash in 0.5ml PBS/PVP	5 min	RT
3. Block: block solution	30 min	RT
4. Secondary Ab: goat anti-mouse IgG2b (Alexa 488)	45 min	37°C
5. Wash in 0.5ml PBS/PVP	5 min	RT, dark
6. Texas Red Phalloidin	30 min	37°C, dark
7. Wash in 0.5ml PBS/PVP	5 min	RT, dark
8. Hoechst 33258	10 min	RT, dark

Primary Ab:	Aurora A (Mab) 1 ul stock + 499 ul block solution	(1:500)
Secondary Ab:	5ul stock (10ug) + 495 ul block solution	(20ug/ml stock)
(gam IgG2b)	250 ul of 20ug/ml stock + 750 ul block solution	(5ug/ml)
Texas Red Phalloidin:	10 ul stock + 240 ul block solution	(8 U/ml)
Hoechst 33258:	10 ul stock + 990 ul block solution	(10ug/ml)

Appendix B: Polyclonal Aurora A Staining Protocol

Triple Staining of mouse oocytes/embryos (Aurora A polyclonal, tubulin, chromatin)

Fixed oocytes/embryos kept at 4 degrees C in block solution at least overnight before starting staining procedure.

		Time	Temp
1.	Primary Ab: rabbit anti Aurora A	60 min	37°C
2.	Wash in 0.5ml PBS/PVP	5 min	RT
3.	Block: block solution	30 min	RT
4.	Secondary Ab: gar IgG frag. (Alexa 594)	45 min	37°C, dark
5.	Wash in 0.5ml PBS/PVP	5 min	RT, dark
6.	Primary Ab: mouse anti a/b-tubulin cocktail (50:50)	60 min	37°C, dark
7.	Wash in 0.5ml PBS/PVP	5 min	RT, dark
8.	Block: block solution	30 min	RT, dark
9.	Secondary Ab: gam IgG1 (Alexa 488)	45 min	37°C, dark
10.	Wash in 0.5ml PBS/PVP	5 min	RT, dark
11.	Hoechst 33258	10 min	RT, dark

Primary Ab:	a-tubulin: 2ul stock + 998 ul block solution	(1:500)
	b-tubulin: 1.5ul stock + 120 ul block solution	(1:500)
	a/b-tubulin cocktail: 200ul a-tubulin + 200ul b-tubulin	(1:1000)

Primary Ab:	Aurora A (Pab) 5 ul stock + 495 ul block solution	(1:100)

Secondary Ab: 5ul stock (10ug) + 495 ul block solution (20ug/ml
stock)
(gar IgG frag.) 250 ul of 20ug/ml stock + 750 ul block solution (5ug/ml)

Secondary Ab: 5ul stock (10ug) + 495 ul block solution (20ug/ml
stock)
(gam IgG1) 250 ul of 20ug/ml stock + 750 ul block solution (5ug/ml)

Hoechst 33258: 10 ul stock + 990 ul block solution (10ug/ml)