Video Article

A Neural Network-Based Identification of Developmentally Competent or Incompetent Mouse Fully-Grown Oocytes

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Abstract

Infertility clinics would benefit from the ability to select developmentally competent *vs.* incompetent oocytes using non-invasive procedures, thus improving the overall pregnancy outcome. We recently developed a classification method based on microscopic live observations of mouse oocytes during their *in vitro* maturation from the germinal vesicle (GV) to the metaphase II stage, followed by the analysis of the cytoplasmic movements occurring during this time-lapse period. Here, we present detailed protocols of this procedure. Oocytes are isolated from fully-grown antral follicles and cultured for 15 h inside a microscope equipped for time-lapse analysis at 37 °C and 5% CO_2. Pictures are taken at 8 min intervals. The images are analyzed using the Particle Image Velocimetry (PIV) method that calculates, for each oocyte, the profile of Cytoplasmic Movement Velocities (CMVs) occurring throughout the culture period. Finally, the CMVs of each single oocyte are fed through a mathematical classification tool (Feed-forward Artificial Neural Network, FANN), which predicts the probability of a gamete to be developmentally competent or incompetent with an accuracy of 91.03%. This protocol, set up for the mouse, could now be tested on oocytes of other species, including humans.

Video Link

The video component of this article can be found at https://www.jove.com/video/56668/

Introduction

Female infertility is a pathology that affects an increasing number of women. According to the World Health Organization, around 20% of couples are infertile, with a 40% due to female infertility. In addition, one third of women undergoing cancer treatments (300,000/year and 30,000/year in the USA or Italy, respectively) develop premature ovarian failure.

A strategy to prevent infertility in cancer patients is the isolation and cryopreservation of ovarian follicles before the oncological treatment, followed by *in vitro* maturation (IVM) of GV oocytes to the MII stage (GV-to-MII transition). The availability of non-invasive markers of oocyte developmental competence would improve the fertilization and developmental processes and the overall pregnancy success[1,2].

Based on their chromatin configuration observed after staining with the supravital fluorochrome Hoechst 33342, mammalian fully-grown oocytes are classified either as a Surrounded Nucleolus (SN) or a Not Surrounded Nucleolus (NSN)[3]. Besides their different chromatin organization, these two types of oocytes display many morphological and functional differences[3,4,5,6,7,8,9], including their meiotic and developmental competence. When isolated from the ovary and matured *in vitro*, both type of oocytes reach the MII stage, and after sperm insemination, develop to the 2-cell stage, but only those with an SN chromatin organization may develop to term[9]. Although good as a classification method for selecting competent *vs.* incompetent oocytes, the main drawback is the mutagenic effect that the fluorochrome itself and, above all, the UV light used for its detection might have on the cells.

For all these reasons, we searched for other non-invasive markers associated with the SN or NSN chromatin conformation that could substitute the use of Hoechst while maintaining the same high classification accuracy. The time-lapse observation of Cytoplasmic Movement Velocities (CMVs) is emerging as a feature distinctive of the cell status. For example, recent studies demonstrated the association between CMVs recorded at the time of fertilization and the capacity of mouse and human zygotes to complete preimplantation and full-term development[10,11].

Based on these earlier studies, we describe here a platform for the recognition of developmentally competent or incompetent mouse fully-grown oocytes[5,6,7,8]. The platform is based on three main steps: 1) Oocytes isolated from antral follicles are first classified based on their chromatin configuration either as a surrounded nucleolus (SN) or a not-surrounded nucleolus (NSN); 2) Time-Lapse images of CMVs occurring during the GV-to-MII transition of each single oocyte are taken and analyzed with particle image velocimetry (PIV); and 3) the data obtained with PIV

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are analyzed with a Feed-forward Artificial Neural Network (FANN) for blind classification[12,13]. We give details of the most critical steps of the procedure designed for the mouse to make it ready available to be tested and used for other mammalian species (*e.g.,* bovine, monkey and humans).

Protocol

All procedures involving animals were approved by the Institutional Animal Care and Use and Ethical Committees at University of Pavia. Animals were maintained under conditions of 22 °C, 60% air humidity and a light/dark cycle of 12:12 h.

1.Ovary Isolation

1. Inject intraperitoneally 2 four-to-eleven week-old CD1 female mice with 10 U of follicle stimulating hormone with a sterile 1 mL insulin syringe.
2. Wait 46-48 h.
3. Weigh the mouse and anesthetize with an intramuscular injection of 50 mg/kg of Zoletil (Tiletamina and Zolazepan cloridrate). Euthanize by cervical dislocation.
4. Grasp the skin covering the abdominal wall using a pair of dissection forceps. Cut the skin and body wall with a pair of scissors and pull open the incision with forceps.
5. Using a pair of tweezers, move the guts aside, pinpoint the uterine horns and localize the ovaries at their extremity.
6. Gently hold the ovary using watchmaker forceps and use scissors to cut its ligament to the uterus. Repeat with the other ovary.
7. Transfer the collected ovaries into a 35-mm cell culture Petri dish containing 1 mL of M2 isolation medium pre-warmed at 37 °C, 5% CO_2 in air.
8. Remove the fat and the oviduct using fine scissors under a stereomicroscope.

2. Isolation of Cumulus Oocyte Complexes

1. Puncture the ovarian surface using sterile tweezers and a 1G sterile insulin needle until antral cumulus oocyte complexes (COCs) are passively released.
2. Collect COCs with more than three layers of cumulus cells using a mouth-controlled hand-pulled Pasteur micropipette (200 µm in diameter) and transfer them into a 300 µL drop of fresh M2 medium.
3. Remove the cumulus cells surrounding the oocytes using a hand-pulled Pasteur micropipette (80 µm in diameter) by gently pipetting in and out for few seconds,
4. Repeatedly transfer oocytes from a 20 µL drop of M2 medium to another one, until cumulus cells are completely eliminated.

3. Chromatin Organization-Based Oocyte Classification

1. Prepare Hoechst 33342 staining solution at a final concentration of 0.05 µg/mL in M2 medium starting from a mother solution of 5 mg/mL diluted in 1x PBS.
2. Place 3.5 µL micro droplets of Hoechst staining solution at the bottom of a 35 mm Petri dish lid.
3. Transfer each single oocyte into a Hoechst staining drop, place the dish onto a pre-warmed (37 °C) heating plate and cover it with a dark lid to avoid light exposition.
4. After 10 min of incubation at room temperature, observe the oocytes with a fluorescence microscope at 10X magnification under UV light (330-385 nm), for no more than 1-2 s.
5. Classify oocytes as a surrounded nucleolus (SN) (**Figure 1A**), if they present a ring of Hoechst-positive chromatin surrounding the nucleolus.
6. Classify oocytes as a not surrounded nucleolus (NSN), if their nucleolus is not surrounded by Hoechst-positive chromatin and shows disperse heterochromatic spots within the nucleus (**Figure 1B**).

4. Neural Network-Based Oocyte Classification

1. Prepare four 2 µL droplets of α-MEM medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 5 mM taurine, 100 U/mL penicillin, 75 µg/mL streptomycin and 23.5 µg/µL sodium pyruvate into a 35-mm glass bottom Petri dish and cover them with pre-warmed (37 °C) and pre-equilibrated mineral oil to avoid medium evaporation.
   NOTE: If using a live cell-screening system, keep the drops at a specific distance by using a drawn grid of 7 mm x 7 mm as a guideline.
2. Transfer 3- 4 oocytes to each drop.
3. Place the glass bottom Petri dish into a live cell-screening system, equipped with time-lapse recording software (see **Table of Materials**), which allows the observation of oocyte maturation in an environment with stable temperature (37 °C) and CO_2 concentration (5%).
4. Open the time-lapse recording software. On the screen a window appears with a live shot of the oocyte on the left-hand side and all the setting buttons on the right-hand side.
5. Select the center of the oocyte to position it at the screen center. Adjust focus if necessary.
6. Select the **Ph** button and set the **DIA Lamp** to 192; the **Exposure time** to **1/125 s**; the **Gain** to **1.99**; and the **Resolution** to **1600 x 1200**.
7. Select **FL2** and set the **DIA Lamp** to **5**; the **Exposure time** to **3 s**; the **Gain** to **2.37**; and the **Resolution** to **1600 x 1200**.
8. Select the **New point** button in order to record the oocyte position within the culture drop. Repeat the same routine for each oocyte within the culture drop.
9. Select **Time** and set the **Acquisition cycle** to **8 min** and the **Total time** to **15 h**.
10. Select **Start time-lapse** to start the recording cycle.
11. Open the MATLAB software and write >>**cell_piv**; and then select the **PIV Tool**.

12. Choose the folder containing the recorded movie and select any of the .jpeg files to load the whole image sequence. For each oocyte, select the region of interest (ROI) by clicking the **Select area tool**.
13. Select **Process PIV** in the File menu. Select the **Viewer Tool** button.
    NOTE: The program will calculate the velocity vectors within the ROI and automatically produces a data file, saved as a .mat file.
14. Select the .mat file to open. Select the button **Select ROI tool** and draw a circle around the outline of the oocyte. Click Save under the File menu.
    NOTE: The vectors in this region are now displayed and the mean magnitude data in the graph window updated for this new ROI.
15. Open the collected mean magnitude data of the whole time-lapse frames recorded into a spreadsheet, with, on the rows, the frame sequence and, on the columns, each single oocyte analyzed.
16. Organize the data of both NSN and SN oocytes into 10 subgroups.
17. Use 9 subgroups to train the feed-forward artificial neural network iteratively (FANN) and 1 subgroup for blind testing.
    1. Repeat another 9 times, changing the blind testing sample (10-fold cross-validation).

18. Open MATLAB, run the custom-made script **main** and, upon request, insert the name of the file with the training data (**train.txt**), the number of NSN and SN oocytes used for training and the time-lapse interval to be analyzed (typically, from frame # 1-2 to frame # 112-113).
19. Press **enter** to perform the training.
20. Insert the name of the file with the testing data (**test.txt**).
21. Open the vector **test_outputs_cyt** in MATLAB workspace.
    NOTE: A window appears on the screen showing, in the first row, the probability to obtain True Positives (TP or True NSN) and False Positives (FP or False NSN) for NSN and SN oocytes, respectively; instead, in the second row, the probability to obtain False Negatives (FN or False SN) and True Negatives (TN or True SN) for NSN and SN oocytes, respectively.
22. Use the formula: (TP+TN)/(TP+FP+FN+TN), to calculate the FANN accuracy, expressed as a percentage.

Representative Results

Figure 2 shows a representative developmentally competent and incompetent oocyte, respectively at the beginning (GV) and at the end (MII) of the IVM procedure. IVM of fully-grown mouse oocytes occurs during 15 h culture. The Time-Lapse observation records the progression of meiosis and detects major meiotic events, including the GVBD and the extrusion of the first polar body.

The analysis and comparison of more than two-hundred developmentally competent *vs.* incompetent oocytes showed time differences in the progression of meiosis. Specifically, in NSN oocytes, GVBD is significantly delayed of 1-2 time-lapse frames, whereas the PB1 extrusion occurs with a 15 frames delay. Despite these differences, the variability is too high to allow single-oocyte classification. For this reason, the procedure was implemented with a mathematical classification tool.

Figure 3 shows a scheme of the mathematical classification tool used, named Feed-forward Artificial Neural Network (FANN). For each oocyte fed through, FANN simultaneously gives the probability that the gamete is a developmentally competent and the probability that it is an incompetent oocyte. In our experiments, the FANN classified mouse oocytes with a mean accuracy of 91.03%.

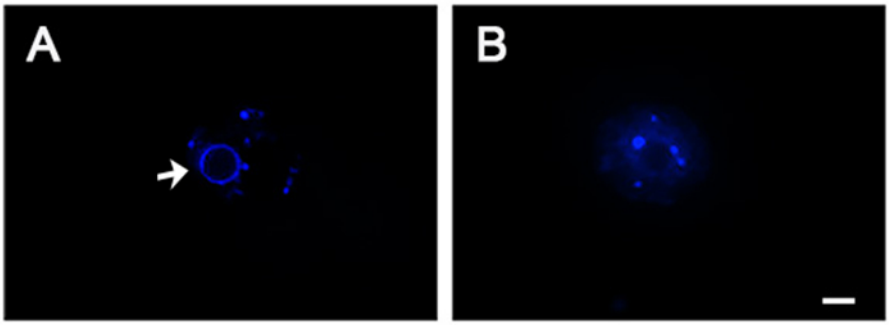

Figure 1. Representative SN and NSN oocytes stained with Hoechst 33342. Following isolation and staining with the fluorochrome Hoechst 33342, fully-grown oocytes are classified either as surrounded nucleolus (SN) (A) or not surrounded nucleolus (NSN) (B), depending on the presence (arrow) or absence, respectively, of a ring of Hoechst-positive heterochromatin surrounding the nucleolus. Scale bar: 5 µm Please click here to view a larger version of this figure.

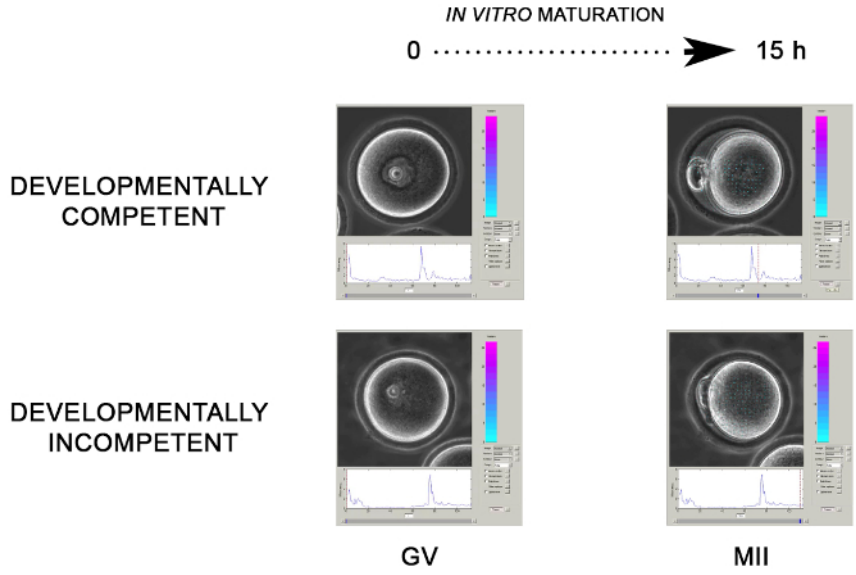

Figure 2. Particle Image Velocimetry analysis of Time-Lapse images. The results of the PIV analysis are shown below the bright-field image for each of the 113 time-lapse frames analyzed. The figure shows the beginning (GV) and the end (MII) of the recording process. Please click here to view a larger version of this figure.

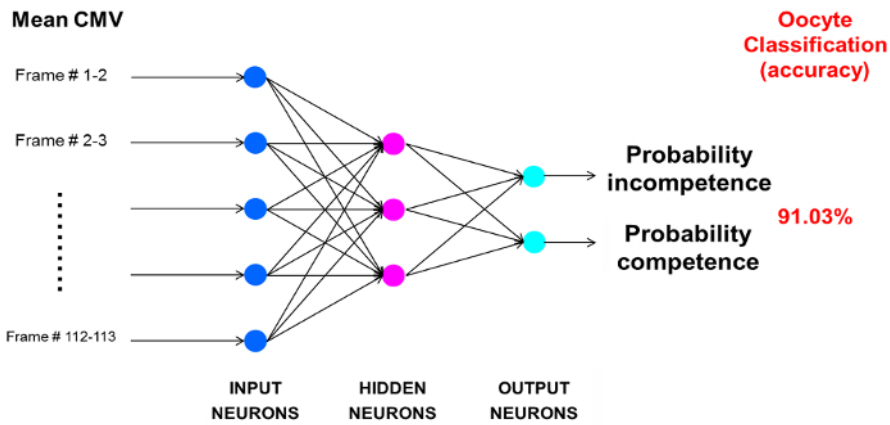

Figure 3. Schematic representation of the Feed-forward Artificial Neural Network. FANN is the mathematical tool used to classify oocytes as developmentally competent or incompetent. Please click here to view a larger version of this figure.

Discussion

There are several critical steps one should take care of while performing this protocol with mouse oocytes as well as with those of other species. Once isolated from their follicles, oocytes should be immediately transferred into the recording drops, as the separation from the companion cumulus cells triggers the beginning of the GV-to-MII transition. A possible modification to the present protocol could be the addition of 3-isobutyl-1-methylxanthine (IBMX) to the M2 medium used for COCs isolation. IBMX prevents the immediate triggering of the GV-to-MII transition and allows a synchronization of the whole experimental group of oocytes.

The live cell-screening system used in our experiments (see **Table of Materials**) limits the number of maturation drops to 4 with 4 oocytes per drop, thus to a total of 16 oocytes per experiment. This limitation could be overcome using other time-lapse live cell-screening systems, commercially available and equipped with several culture chambers.

Based on our experience, the recording interval between a frame and the following is a critical point. After a number of preliminary experiments, we took images every 8 min because this was the minimum time window that allowed the clear observation of the major events occurring during the GV-to-MII transition, such as the germinal vescicle breakdown and the extrusion of the first polar body. This time window might need to be revaluated when observing oocytes of other species.

A drawback of this method is the culture of oocytes in the absence of their surrounding cumulus cells, as the latter are known to further improve the GV-to-MII transition. To this regard, our laboratory is now testing a slight modification to the reported protocol by including the culture of cumulus-free oocytes upon a cumulus cells feeder layer.

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By its nature, the FANN has a fixed number of both input (the number of analyzed CMV modules) and output (either a developmentally competent or incompetent oocyte) neurons, but the number of hidden neurons is chosen by the investigator through a trial and correction approach to obtain the best prediction performance. In our case, we experimented with different numbers of hidden neurons and found that three gave the best results. Also, FANN analyses require a large number of input data (in our experiments 112 CMV modules) to obtain a robust statistics. A possible improvement that could reduce the number of necessary input data is the use of alternative classification tools, such as Support Vector Machine, Tree-bag, or KNN classifiers. When the FANN analysis demonstrates its robustness, step 1 could be eliminated from the procedure.

This protocol, set up for the mouse, could be tested on oocytes of other species including humans. Furthermore, the combination of time-lapse recording with PIV and neural network analyses could be exploited for the observation of CMVs in zygotes[10,11] and in preimplantation embryos for the evaluation of their further developmental potential.

Disclosures

The authors have nothing to disclose.

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