Institute of Animal Physiology and Genetics CAS, v. v. i.
Rumburská 89, 277 21 Liběchov, Czech Republic
tel.: +420 315 639 532; fax: +420 315 639 510
E-mail: uzfg@iapg.cas.cz; URL http://www.iapg.cas.cz

Application to the Matousek Award 2020 of IAPG CAS
Final report

Name of applicant: Dávid Drutovič
Name of project: The development and optimization of live-cell imaging of mouse zygotes and human oocytes using light-sheet microscopic technology
Laboratory: Laboratory of DNA integrity

The main goal of the proposed project was to optimize live-cell imaging of mouse preimplantation embryos to successfully imaged complete development from zygote to blastocyst and to established an experimental system for live-cell multi-color fluorescence microscopy of human vitrified/thawed oocytes. Understanding the process of chromosome segregation in the mammalian oocytes or zygotes and why human oocytes are more prone to segregation errors is a fundamental question relevant to human health. Here we combined mouse zygotes, human oocytes, and a unique Viventis LS1 Live inverted light-sheet microscope system specifically developed for long time-lapse live-cell imaging of oocytes and zygotes to better study dynamic processes of chromosome segregation and spindle formation in live cells. The confocal live-cell imaging has been tested successfully in mammalian oocytes. Still, its use for live imaging in mice to study early embryonic development has been limited because of a relatively slow process of image acquisition and phototoxic damage - none of the studies of early embryo developments using confocal live-cell imaging successfully imaged complete development from zygote to blastocyst. We aimed to optimize successful imaging of full development from zygote to blastocyst in mice and human oocyte meiosis using an inverted light-sheet microscope.

The principal goals and experimental plans were as follows:

1. Optimization of live-cell imaging of mouse zygotes

To optimize the imaging of preimplantation development into the blastocyst using a light-sheet microscope, we first compared the development in a medium designed for culturing of mouse embryos (EmbryoMax KSOM Mouse Embryo Media, Millipore, Merck, catalog number MR-020P-F) and in a medium intended for culturing human embryos in assisted reproductive technology cycles (Global, Cooper Surgical, catalog number LGGG-050). To visualize the chromosomes, we microinjected histone H2B-mCherry mRNA into mouse zygotes. Whereas overexpression of histone H2B may affect the fidelity of embryonic development, we tested two different concentrations – 50 ng/ul – concentration standardly used for mouse oocyte microinjection and one lower concentration – 35 ng/ul.

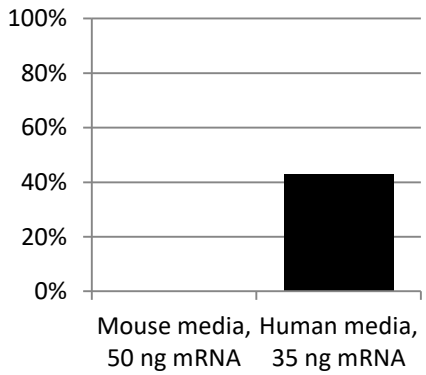

Fig. 1 *Comparison of development into the blastocyst stage in different culture conditions (medium designed for culturing mouse embryos and culturing human embryos in assisted reproductive technology cycles) and different concentrations of H2B-mCherry mRNA (35 vs. 50 ng/ul).*

Mouse zygotes cultured in mouse embryo development media and microinjected with 50 ng/µl H2B-mCherry mRNA did not develop into the blastocyst stage (0%)(Fig. 1), whereas 43% of zygotes cultured in human assisted reproduction medium and microinjected with 35 ng/ul H2B-mCherry developed into the blastocyst stage (Fig. 1, 2). These results indicate that a human medium with lower concentrations of H2B-mCherry mRNA is suitable for imaging the preimplantation development of mouse embryos.

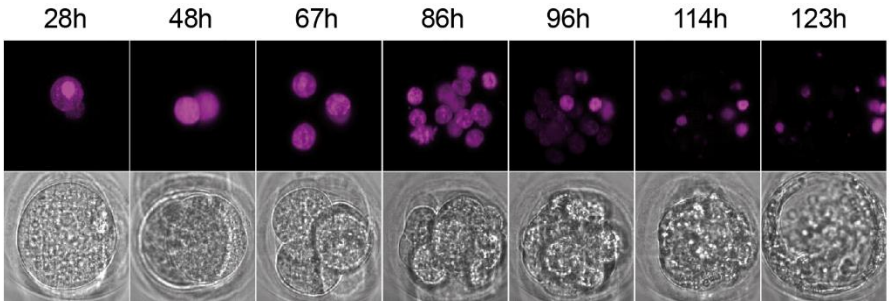

Fig. 2 *An illustrative image of blastocyst development over time after hormonal stimulation imaged every 10 min. H2B-mCherry (magenta) and bright-field. Time after hCG stimulation (hours).*

In the next step, we focused on improving the culture conditions. For this reason, we reduced the presence of oxygen during scanning to 5%. Human embryos were usually cultured in 20% oxygen. The previous data from animal studies reported that cleavage-stage embryos are exposed to an oviductal oxygen concentration of 7%. We hypothesized that mouse zygotes cultured *in vitro* under 20% oxygen are exposed to oxygen at nonphysiologic concentrations, increasing the production of reactive oxygen species and cause oxidative damage. We also looked at whether the length of the imaging would affect the development of the blastocyst. The main phase of genome activation occurs during the two-cell stage. We hypothesized that starting imaging after genome activation (from the 2-cell stage) could improve blastocyst development. To study this, we imaged early mouse embryos microinjected with 35 ng/ul H2B-mCherry mRNA from either the 1-cell stage - zygote or the two-cell stage. 55% of early mouse embryos imaged from the two-cell stage developed into the blastocyst (n=18), while only 45% of embryos imaged from the zygote (n=9). Comparing the percentage of embryos with the segregation error did not show significant differences between the two groups. The results show that reducing the oxygen tension to 5% improved the development into the blastocyst. When monitoring development into the blastocyst, embryos scanned from the two-cell stage develop better.

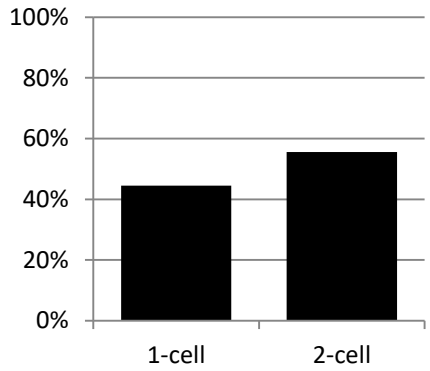

Fig. 3. *Comparison of embryonic development to the blastocyst stage from the "1-cell" mouse zygote or the two-cell stage.*

To further improve blastocyst development, we decided to try using embryous isolated from transgenic H2B-GFP mice. Their advantage is that there is no need to use a microinjection technique that causes stress to the embryos and can worsen development prognosis. We used a reduced oxygen tension of 5% in the culture in a medium designed for culturing human embryos in assisted reproductive technology cycles and imaged the embryos from the two-cell stage. 84% of H2B-GFP transgenic early mouse embryos developed into a blastocyst (n=13) (Fig. 4). These results suggest that the best development into the blastocyst can be achieved by scanning embryos from transgenic mice.

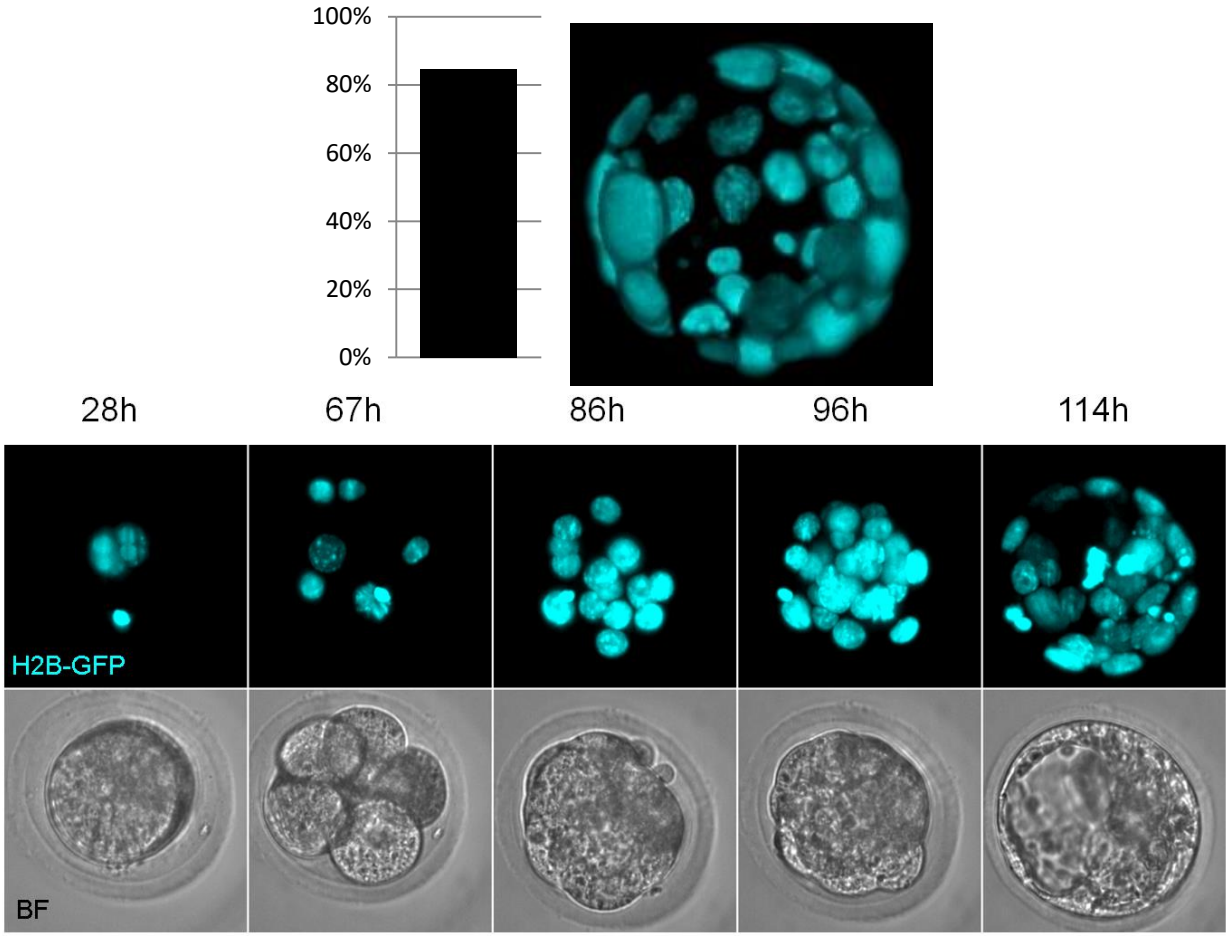

***Fig. 4**. Percentage development into the blastocyst of H2B-GFP transgenic early mouse embryos. 3D image of a blastocyst reconstruction. Development of embryo at different times after hCG stimulation. H2B-GFP (cyan). Bright-field (BF).*

2. Development of live-cell imaging of human vitrified/thawed oocytes

Mouse oocytes serve as a valuable model system for analyzing the intracellular dynamics during cell division. However, some signaling pathways could function differently in mouse and human oocytes. Therefore, it is critical to validate the research data obtained from experimentation in the mouse model directly in humans. Nevertheless, the availability of human oocytes for research is limited and requires close collaboration with a fertility clinic.

Immature human oocytes exhibiting normal morphology and intact prophase nucleus after retrieval were kept in culture for 6-8 hours. Only maturation-competent cells, capable of resuming meiosis, were individually cryopreserved using clinically proven vitrification protocol (KitazatoTM). The experienced embryologist has performed vitrification procedures directly in clinical settings to avoid possible disturbance during live cells' transport. The collected samples were transferred to a research laboratory in liquid nitrogen. Prior experiment, vitrified oocytes were warmed using Kitazato Thawing Media according to the manufacturer's protocol and short incubated in Continuous Single Culture Complete medium supplemented with HSA in an incubator with 5% CO2 atmosphere and 37 °C temperature.

To study dynamic processes of spindle assembly and chromosome segregation in human oocytes, we aimed to established a live-cell imaging system of vitrified/thawed human oocytes completing maturation in vitro. First, we microinjected human oocytes with 100 ng/ul H2B-mCherrry and cultured them in 100 nM SiR-tubulin for microtubule visualization. In this experiment, around 71% of oocytes extrude the first polar body, but we observed only a low signal of microtubules. Whereas we usually used 100 nM SiR-tubulin for microtubule visualization in mouse oocytes, we hypothesized that it was caused by decreased permeability of human oocytes zona pellucida. In the next step, we microinjected human oocytes with 100 ng/ul H2B-mCherrry and 250 ng/ul microtubule-associated protein MAP4-EGFP as a marker of

microtubules. Whereas we could visualize spindles, the percentage of oocyte extruded first polar body decreased to around 57%, which a combination of two lasers lines could cause. Therefore we tested a method for live imaging of chromosomes labeled with two different concentration of SiR-DNA, which combine minimal cytotoxicity with excellent brightness and requires no RNA microinjection into oocytes. Notably, 100% of oocytes stained with 75 nM of SiR-DNA (concentration standardly used for mouse oocytes in our experiments) extruded the first polar body (compared to only 66% with 150 nM SiR-DNA), and chromatin was visualized. In the next step, we tried to optimize the two-color imaging of human oocytes.

Rewarmed immature oocytes were microinjected with Map4-Egfp cRNA, and stained with 75 nM SiR-DNA. Significantly, all analyzed oocytes form a bipolar spindle, and around 85% extrude the first polar body, indicating that the imaging does not perturb meiotic progression (Fig. 5). These results describe the imaging as a proper tool for visualizing spindle assembly in live human oocytes.

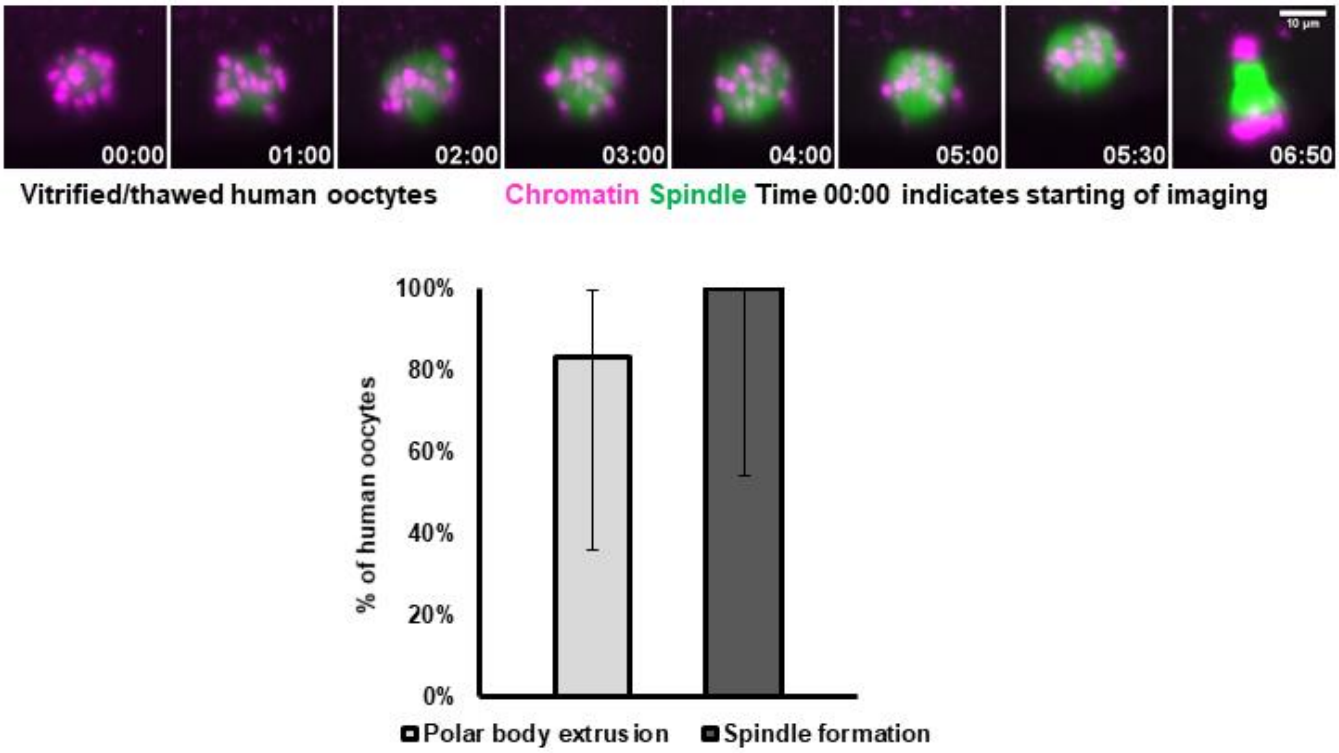

Fig. 5 *Representative images of meiotic maturation in human oocytes. (A) Live cell light-sheet imaging of vitrified/thawed human oocytes expressing MAP4-EGFP (spindle, green) and stained with SiR-DNA (chromatin, magenta). Scale bar 10 µm. (B) Data in (A) was used to quantify % of oocytes formed bipolar spindle or extruded first polar body. The graph shows the mean ± 95% exact confidence intervals. n = 6 oocytes.*

Total estimated project costs – 140 000 CZK: fluorescence probes - SiR-tubulin, SiR-DNA (Spirochrome) – 20000 CZK; oocytes microinjection - microinjection and fixation capillaries – 20000 CZK, loading tips – 10000; oocyte and zygotes cultivation – 40000 CZK; preparation and purification of RNA for microinjection – 30000 CZK. Light-sheet microscopy – chambers – 20000 CZK

Total project costs – 135 362,93 CZK: fluorescence probes – 29 346,86 CZK; mouse zygotes cultivation – 35 881.91; human oocyte thawing and culture - 55 034,14 CZK; Light-sheet microscopy – chambers – 15 100.02 CZK

Summary:

This project helped us optimize imaging of mouse embryos and human vitrified/thawed oocytes using light-sheet microscopy. We tested different culture conditions of mouse zygotes and described that culture in media designed for human embryous and reduced oxygen levels improve development to the blastocyst stage. This condition, combined with a low concentration of microinjected RNA, should be used to imaging mouse zygotes into the blastocyst stage in a high spatial and temporal resolution. We also described the

unique two-color imaging of human vitrified/thawed oocytes using light-sheet technology, which will be used for the project focused on spindle formation and chromosome segregation in human oocytes. We plan to use these imaging techniques in two papers focused on the regulation of mitotic division of early mouse embryos (Chk1 kinase monitors the level of DNA replication in mice zygotes – first author Tomas Duricek, last author David Drutovic; Maternally expressed CHK1 kinase and CDC25A phosphatase control cell cycle progression in early embryonic development in mouse to ensure genome integrity protection and zygotic genome activation– first author Lucie Knoblochova, last author David Drutovic). In cooperation with Dr. Holubova, we plan to publish the methodological paper in a high-impact journal focused on a unique combination of vitrified/thawed human oocytes, light-sheet imaging, and new fluorescence probes. In summary, the combination of cutting-edge equipment, the concentration of exclusive expertise in live-cell imaging techniques, access to rare biological samples (human oocyte), and here described imaging development and optimization make us well-positioned to tackle mechanisms behind spindle assembly in human oocytes and meiotic-to-mitotic transition in mouse embryos.

Libechov, 7 May 2021

Dr. David Drutovic, Ph.D.

Principal investigator

By my signature, I am confirming that I fully agree with a reservation with the way of implementation and results achieved within the project."

Libechov, 7 May 2021

Dr. David Drutovic, Ph.D.

Temporarily Head of the Laboratory