# Live imaging of cortical granule exocytosis reveals that *in vitro* matured mouse oocytes are not fully competent to secret their content

Andrea I. Cappa[1], Matilde de Paola[1], Paula Wetten[1], Gerardo A. De Blas[1,2], Marcela A. Michaut[1,3]

**Affiliations**

[1]Instituto de Histología y Embriología, Universidad Nacional de Cuyo- CONICET, Av. Libertador 80, 5500, Mendoza, Argentina.

[2]Facultad de Ciencias Médicas, Universidad Nacional de Cuyo

[3]Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Cuyo.

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**Corresponding author:** Dr. Marcela A. Michaut. Laboratorio de Biología Reproductiva y Molecular. Instituto de Histología y Embriología, Universidad Nacional de Cuyo-CONICET. Av. Libertador 80. 5500 Mendoza. Argentina. Phone: 54-261-4135000 ext 2759. Fax 54-261-4494117. E-mail: mmichaut@gmail.com

# Summary statement

Live imaging of cortical granule exocytosis reveals that *in vitro* matured mouse oocytes have a severely reduced response to strontium chloride

# Abstract

Oocyte *in vitro* maturation does not entirely support all the nuclear and cytoplasmic changes that occur physiologically and it is poorly understood whether *in vitro* maturation affects the competence of cortical granules to secret their content during cortical reaction. Here, we characterize cortical granule exocytosis (CGE) in live mouse oocytes activated by strontium chloride using the fluorescent lectin FITC-LCA. We compared the kinetic of CGE between ovulated (*in vivo* matured, IVO) and *in vitro* matured (IVM) mouse oocytes. Results show that: 1) IVM oocytes have a severely reduced response to strontium chloride; 2) the low response was confirmed by quantification of remnant cortical granules in permeabilized cells and by a novel method to quantify the exudate in non permeabilized cells; 3) the kinetic of CGE in IVO oocytes was rapid and synchronous; 4) the kinetic of CGE in IVM oocytes was delayed and asynchronous; 5) cortical granules in IVM oocytes show an irregular limit with the cortical granule free domain. We propose the analysis of CGE in live oocytes as a biological test to evaluate the competence of IVM mouse oocytes.

# Introduction

In mammalian oocytes, cortical reaction, also named cortical granule exocytosis (CGE), is a fundamental process in which the cortical granules fuse with plasma membrane after sperm fertilization preventing polyspermy and ensuring embryo development (reviewed by (Liu, 2011; Sun, 2003). The production of cortical granules in mammalian oocytes is a continuous process, and newly synthesized granules are translocated to the cortex until the time of ovulation (Ducibella et al., 1994). The migration of cortical granules to the cortex is mediated by microfilaments (Cheeseman et al., 2016; Connors et al., 1998) and is an important step in cytoplasmic maturation (Ducibella et al., 1988a). The localization of cortical granules in the cortical region is used routinely as a criterion in assessing the maturity and organelle organization of developing oocytes (Damiani et al., 1996).

Oocyte meiotic maturation is a complex process that involves coordinated nuclear and cytoplasmic changes and is defined as the resumption and completion of the first meiotic division to the progression until metaphase II. The completion of nuclear and cytoplasmic processes defines the competence of an oocyte. Only a competent oocyte can be fertilized and support the early embryo development (Li and Albertini, 2013). Underlying cellular and molecular mechanisms of mammalian oocyte maturation is still poorly understood and is under continuous investigation (Reader et al., 2017).

*In vitro* maturation (IVM) is a culture method that allows germinal vesicle (GV) oocytes to undergo IVM until reaching metaphase II stage (MII oocytes). IVM is used in both animal and human assisted reproduction but the reproductive efficiency is very low.

Cortical granules become fully competent for exocytosis after completion of the first meiotic division in MII oocytes (Ducibella et al., 1988b; Ducibella and Buetow, 1994).

How IVM affects the competence of cortical granules to secret their content is under continuous investigation. In this report, we investigated the capacity of reacting to strontium chloride ($SrCl_{2)}$ of *in vivo* (IVO) and *in vitro* matured (IVM) oocytes, using a fluorescent method to analyze CGE in real time.

# Results

**The dynamics of cortical reaction can be evaluated in real time by LCA-FITC**

The distribution of cortical granules in rodents MII oocytes has been demonstrated using fluorescence microscopy with the fluorescently labeled lectin *Lens culinaris* agglutinin (LCA) (Cherr et al., 1988; Ducibella et al., 1988a). Cherr and collaborators demonstrated that LCA allows the localization of cortical granule content before and after exocytosis in hamster MII oocytes (Cherr et al., 1988). LCA-FITC has affinity to alpha-mannose residues present in the content of cortical granules. When this content is secreted during CGE, the secretion can be detected by fluorescence microscopy. Hence, we use LCA-FITC to analyze CGE in real time. First, we attempted to activate CGE with mouse sperm by in vitro fertilization. Unfortunately, this method was impracticable because mouse sperm agglutinated in presence of LCA-FITC (see Movie S1). Then, we decided to activate CGE parthenogenetically with $SrCl_2$. This parthenogenetic activator has several advantages compared to other chemical and physical activators: its use is very simple, it is not toxic for the cell, it mimics the natural pattern of calcium waves after sperm penetration, and synchronizes cortical reaction in most of the oocytes within a relatively short time frame. This is a significant advantage over using fertilized eggs, which are typically activated at various times throughout an experiment. In addition, artificial activation alleviates concerns of having to differentiate sperm-derived and egg-derived constituents.

We incubated IVO oocytes in the presence of LCA-FITC to detect the secretion of cortical granules into the perivitelline space by the increase of fluorescence. The activator was present in the incubation media during the entire experiment. Only MII oocytes showing the first polar body were used for the assay. As shown in Fig 1A (upper panel), we were able to detect a visible increase of LCA-FITC intensity after 10-20 minutes of the addition of strontium chloride (see also Movie S2). The fluorescence´s intensity was irregular. It drew a fluorescent semicircle with high intensity and a small portion of the circle with low intensity, resembling the localization of cortical granules (vegetal pole) and the cortical granules free domain (animal pole), respectively (Fig 1B, compare upper and lower panel for 50 min, also see Fig. 2A, IVO panel).In contrast, control (not activated) MII oocytes did not show any fluorescence´s increase during the recorded time (lower panel in Fig. 1A and 1B, and Movie S3).

According with the literature, the observed time correlates with the timing of CGE in MII mouse oocytes (Liu, 2011). To demonstrate that the fluorescence´s increase corresponded

effectively to the release of cortical granules, the cells were pooled and fixed for cortical granules quantification as we previously described (Bello et al., 2016; de Paola et al., 2015). After fixation, cells were permeabilized and the remnant of cortical granules was stained with LCA-rhodamine. Using the program Image J, the density of cortical granules (in the same oocytes analyzed in live condition) was quantified as a measurement of cortical reaction. As predicted, the density of remnant cortical granules (represented as CG/100 $\mu m^2$) was significantly lower in oocytes treated with $SrCl_2$ than in control oocytes (Fig 1C). This result indicates that cortical granules fused with plasma membrane and secreted its content into the perivitelline space.

Cherr and collaborators have demonstrated by transmission electronic microscopy that gold-LCA binds to the microvilli of the activated zona-free hamster eggs (Cherr et al., 1988). In fact, as a consequence of the activation of live MII oocytes in presence of LCA-FITC, the exterior of the cells was also stained by this fluorescent lectin after cell fixation. We observed by confocal microscopy that LCA-FITC (used to stain the secretion of cortical granules in live oocytes), also drew a punctuate pattern in the outside of the fixed oocytes. This punctuate pattern was suitable to be quantified in a similar manner to cortical granules quantification. We named this pattern 'exudate dots'. We quantified the dots´ density and found that this punctuate pattern can also be used to measure the magnitude of cortical reaction (Fig 1D y 1E). Figure 1E shows representative images for control (left column, IVO, not activated) and activated (right column, IVO $SrC_2$,) oocytes. In control condition, cortical granules remained inside of the cell and no cortical reaction is observed. Thus, the upper panel of figure 1E (left) shows cortical granules stained with LCA-rodhamine in a control oocyte (CG "inside"). Without activation and incubated in presence of LCA-FITC, no cortical reaction is observed. This is evidenced by the absence of LCA-FITC dots in the exudate of the same oocyte (lower panel, left, exudate "outside"). On the contrary, in the activated condition, the content of cortical granules was secreted and stained by the fluorescent LCA during live imaging. The upper panel of figure 1E (right) shows the remnant cortical granule after strontium activation (CG "inside") and the exudate´s dots stained with LCA-FITC (lower panel, exudate "outside") for the same oocyte. Finally, to validate LCA-FITC staining in a physiological context, we performed in vitro fertilization with a modified protocol to avoid spermatozoa agglutination. Oocytes were inseminated with capacitated spermatozoa in absence of LCA-FITC. After 2 hours, embryos were transferred to an LCA-FITC-supplemented medium and incubated for additional 5 hours. As shown in Figure S1, in vitro fertilized oocytes showed a similar pattern of exudate in live and fixed cells similar to the pattern observed in strontium

activated oocytes. Altogether, these experiments demonstrate that the increase of fluorescence´s intensity corresponds to the secretion of cortical granule content. Hence, the dynamics of CGE can be evaluated in live cells by the analysis of the increase of LCA-FITC fluorescence´s intensity accumulated in the perivitelline space through time. Additionally, this approach also allows quantifying exudate dots in non permeabilized oocytes after live imaging.

**Live imaging of cortical reaction in ovulated and *in vitro* matured mouse oocytes**

Injection of germinal vesicle- intact oocytes (GV oocytes) with mRNA is routinely used to study the role of different proteins in oocyte maturation. However how closely in vitro maturation resembles the in vivo process and what the impact of in vitro maturation is on cortical granules releasing capacity remains unexplored. To analyze whether in vitro maturation has any impact on CGE, we *in vitro* matured mouse oocytes in two different media: CZB, a regular medium used for *in vitro* mouse oocyte maturation, and G-IVF, a medium used for human oocytes. Only cumulus-oocyte complexes were selected for IVM assays. First, we compared the morphology of IVM oocytes in either CZB or G-IVF medium with IVO oocytes. Results showed that both media supported *in vitro* oocyte maturation in a similar percentage. The percentage of IVM oocytes, quantified morphologically by the extrusion of the first polar body, was 64.4 ± 14.49 % in CZB (n = 97) and 74.5 ± 7.08 % in G-IVF (n = 325). Next, we assayed CGE in live cells using the method described in the previous section. We incubated IVO and IVM oocytes in the presence of LCA-FITC and analyzed the increase of fluorescence´s intensity in the perivitelline space after triggering CGE with $SrCl_2$ during 60 minutes. As shown in Fig 2A, IVO oocytes initiated CGE before IVM oocytes. CGE in IVO oocytes started around 16 minutes after $SrCl_2$ addition (16 ± 6 min, see IVO condition in Fig 2B and Movie S4). Surprisingly, only a low percentage of IVM oocytes were able to respond to the parthenogenetic activator. Figure 2C shows that only 13 % of oocytes matured in CZB and 14 % of oocytes matured in G-IVF medium were able to respond to $SrCl_2$ (see Movie S5 y S6), while 92 % of IVO oocytes responded to the stimulus. These percentages were significatively different compared to responding IVO oocytes ($p<0.0001$, test of difference between two proportions). In addition, IVM delayed the start time of CGE (Fig 2A and 2B). IVM oocytes responded around 30-35 minutes after the addition of strontium chloride

(31 ± 11 minutes in CZB and 34 ± 16 minutes in G-IVF).We wondered whether those oocytes that did not respond within 60 minutes would be activated in a prolonged assay.

When the live imaging was extended until 120-180 minutes, we observed that IVM oocytes were not activated by $SrCl_2$ (absence of cortical reaction and parthenogenetic development; data not shown). These results indicate that most IVM oocytes are not able to respond to $SrCl_2$. Figure 2D shows the kinetic of cortical reaction for IVO and IVM oocytes. IVO oocytes showed a rapid and synchronous response to $SrCl_2$. However, IVM oocytes presented a later and asynchronous response to the activator of CGE (Fig 2D). This asynchronous response is evidenced, mathematically, by a wider standard deviation in the start time of CGE in IVM oocytes (in both media) compared to IVO oocytes (Fig 2B).

Then, after each experiment, cells were pooled at the end of the imaging session and fixed for cortical granule staining with LCA-rhodamine. The magnitude of CGE was analyzed in the whole cohort of imaged oocytes by quantification of density of remnant cortical granules. Figure 3A shows that IVO oocytes responded in a higher magnitude that IVM cells (compare blue, pink and red bars). In effect, IVO oocytes secreted around 40% of cortical granules when stimulated by $SrCl_2$ (blue bar), while IVM oocytes only released 17% (CZB IVM, pink bar) and 25% (G-IVF IVM, red bar) of granules. To better understand oocyte´s distribution, cells were grouped according to similar cortical granule density and plotted as histograms (Fig 3B, D and F). In control conditions, most of the cells had more than 20 CG/100 $\mu m^2$ (see gray bars and gray line in Fig 3B and 3C, respectively). After stimulation with $SrCl_2$, 70% of IVO oocytes had less than 20 CG/100 $\mu m^2$ (average for IVO oocytes) (see blue bars in Fig 3B and blue lines in Fig 3C).This means that most of the cells responded to the activator and secreted the content of cortical granules. In contrast, when IVM oocytes were stimulated, only 20-30% of cells showed less than 20 CG/100 $\mu m^2$ (see Fig 3D-E and 3F-G), indicating that most of IVM oocytes were not able to secret cortical granule´s content. Similar results were obtained by quantifying exudate dots in the same oocytes (Fig 4). Figure 4B shows that the exudate in non stimulated IVO oocytes (control condition, gray bar) has between 0-2 exudate dots/100 $\mu m^2$. On the contrary, when IVO oocytes were activated with $SrCl_2$, around 80 % of the cells showed more than 4 exudate dots/100 µm2. Then, we considered this number as the minimum cortical granule´s density for an activated oocyte. When IVM oocytes were analyzed, both IVM conditions -CZB and G-IVF medium-, showed that 70% of the cells had less than 4 exudate dots/100 $\mu m^2$, indicating that most of the IVM oocytes were not activated by $SrCl_2$ (see Fig 4C and 4D).

It is known that localization of cortical granule is mainly cytoplasmic in GV oocytes and during IVO maturation they migrate to the cortical region of MII stage. This migration and final localization determines that MII oocytes have two poles easily distinguishable: the

cortical granules region (also named vegetal pole) and the cortical granules free domain (also named animal pole) (de Paola et al., 2015; Ducibella et al., 1988a). We wondered whether IVM may perturb cortical granule migration and compared cortical granules localization between IVO and IVM oocytes using 3D reconstruction from multiple images. Results showed that in both conditions cortical granules localized at cortical region; however, the limit between the two poles was different. IVO oocytes had a sharp and defined boundary (Fig 4E, IVO panel; sharp limit in 16 from 23 oocytes), while IVM oocytes showed an irregular limit with the cortical granule free domain (Fig. 4E, CZB panel, sharp limit in 0 from 7 oocytes; G-IVF panel, sharp limit in 4 from 19 oocytes).

These results suggested that IVM may also perturb the molecular mechanism involved in the correct migration and/or localization of cortical granules.

# Discussion

In this study, we evaluated CGE in real time using the fluorescent lectin FITC-LCA. A similar approach has been reported by Satouh and collaborators (Satouh et al., 2017). We investigated the capacity of reacting to $SrCl_2$ of IVO and IVM oocytes. We found that, even when IVM oocytes had a normal morphology, they responded in a very low percentage compared to IVO oocytes. The low response was confirmed by quantification of remnant cortical granules in permeabilized cells and by a novel method to quantify the exudate dots in non permeabilized cells. The kinetic of CGE in IVO oocytes was rapid and synchronous. On the contrary, it was delayed and asynchronous in IVM oocytes. Cortical granules´ distribution in IVM oocytes shows an irregular limit with the cortical granule free domain.

Why in vitro maturation alters the competence of cortical granules to secret their content is still an unanswered question. The membrane fusion during this particular secretory process in mouse oocyte is thought to be mediated by SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) and other regulatory proteins. In effect, it has been shown that the SNAREs SNAP-25 (Ikebuchi et al., 1998) and Sintaxina 4 (Iwahashi et al., 2003) are involved in cortical granule exocytosis in IVO mouse oocytes. We have demonstrated that the alpha-SNAP/NSF complex regulates membrane fusion during cortical reaction and we have proposed a working model for cortical reaction in IVO mouse oocytes (de Paola et al., 2015). In addition, we also have characterized the participation of Rab3A in the cortical reaction of IVO mouse oocytes. Whether in vitro maturation disturbs the function of proteins involved in cortical granule exocytosis has not been yet investigated. Tsai and collaborators have shown

that maturation-dependent migration of SNARE proteins, clathrin, and complexin to the porcine oocyte's surface blocks membrane traffic until fertilization (Tsai et al., 2011). Nevertheless, this general conclusion was reached using IVM oocytes. To the best of our knowledge, this is the first work that compares CGE in real time between IVO and IVM oocytes. Our findings invite to review the results and conclusions of the literature obtained with IVM oocytes since they should not be extrapolated to IVO oocytes.

The development of mouse oocyte cortical reaction competence during oocyte maturation between GV oocytes (incompetent) and MII oocytes (competent) is accompanied by morphological changes in cortical vesicles (Ducibella et al., 1988b). These changes have been associated with the correct maturation of calcium reservoirs such as endoplasmic reticulum (Kim et al., 2014; Latham, 2015), mitochondria (Dumollard et al., 2006), and probably cortical granules. Thus, calcium physiology and its reservoirs need to be explored in IVM oocytes in detail.

The localization of cortical granules in the cortical region is used as a criterion in assessing the maturity and organelle organization of developing mouse oocytes (Damiani et al., 1996). This work shows that cortical granules in IVM oocytes are localized in the cortical region; nevertheless, the limit between the animal and vegetal poles was different between IVO and IVM oocytes. While for IVO oocytes the boundary limit of cortical granules was well defined in 70 % of cells, for IVM oocytes, this limit was sharp only for 0-21% of cells. It would be interesting to analyze the relationship between the irregular boundary of cortical granules and the successful activation of the oocyte. However, considering our findings show that IVM oocytes are not fully competent for cortical granules exocytosis, this analysis would only be possible having a transgenic mouse which *in vivo* expresses a fluorescent molecular marker on cortical granules.

Here, we are reporting evidences that demonstrate that the morphological observations alone are not a sufficient criterion to determine the competence of cortical granules and, in consequence, the oocyte´s competence. Cortical reaction has been suggested as an oocyte quality indicator in pikeperch (Zarski et al., 2012). Satouh and collaborators have shown that the use of LCA-FITC is innocuous for pregnancy and delivery of mouse pups (Satouh et al., 2017). In this work, we have demonstrated that the pattern of LCA-FITC staining is similar in in vitro fertilized and parthenogenetic activated oocytes. Therefore, we propose the analysis of CGE in live mouse oocytes as a biological and innocuous test to determine the competence of a mouse oocyte (or early embryo). Knowing that IVM media does not entirely support all

the nuclear and cytoplasmic changes that occur physiologically, this biological test would also allow evaluating the capability of maturation media for supporting *in vitro* maturation.

# Materials and methods

The protocol was approved by the Institutional Animal Care and Use Committee of the School of Medicine of the National University of Cuyo (Protocol approval 24/2014 and 52/2015).

### Reagents

All chemicals, unless stated otherwise, were purchased from Sigma-Aldrich Chemical Inc. (St. Louis, USA). All solutions were prepared with sterile and apyrogenic distilled water Roux-Ocefa (Bs. As., Argentina). G-IVF ™ PLUS and EmbryoMax® CZB were from Vitrolife (Sweden) and Merk Millipore (USA), respectively. Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were a generous gift from Syntex (Argentina).G-IVF, CZB and M16 medium drops (with/without milrinone) were always covered with mineral oil and gassed overnigth at 37°C in a humidified atmosphere containing 5% (v/v) $CO_2$ before use.

### Immature oocyte (GV) collection

All oocytes were obtained from CF-1 female mice between 6 and 12 weeks old. For follicular growth stimulation, female were injected with 10 IU PMSG (Syntex, Argentina). After 43-45 h of PMSG injection, immature cumulus-oocyte complexes (COCs) were collected from ovary in MEM/ HEPES (Minimum Essential Medium with 100 µg/ml sodium pyruvate, 10 µg/ml gentamicin, 25 mM HEPES pH 7.3) supplemented with milrinone (2.5 µM), which inhibits oocyte maturation. Then they were incubated, in the presence of milrinone, in drops of G-IVF or CZB medium until *in vitro* maturation.

### Mature oocyte (MII) collection

Females were injected with 10 IU hCG (Syntex, Argentina) after 48 h of PMSG injection. MII oocytes were collected from ampulla 15-17 h later in MEM/HEPES medium. Cumulus cells were removed with a brief exposure to 0.04% hialuronidase and the oocytes were incubated in drops of M16 until partenogenetic stimulation.

### *In vitro* maturation

COCs were washed through three drops of either G-IVF or CZB medium without milrinone. Then, they were incubated during 15-17 h in a final drop of 100 µl of the same medium.

### Cortical reaction in real time

Only oocytes with polar body were considered. In the case of *in vitro* matured oocytes, they were first devoided of cumulus cell by pipetting. No more than 10 MII oocytes were selected for each condition. They were quickly washed in two drops of $Ca^{2+}$-free M2 medium supplemented with 25 µg/ml *Lens culinaris* Agglutinin (LCA)-FITC (Vector Laboratories, Burlingame, CA) with/without 30 mM $SrCl_2$. Then, they were placed in a round chamber containing a final drop of 50 µl of the same solution covered with mineral oil. Immediately, images were taken at 37ºC every minute during 1 h using an inverted Eclipse TE300 Nikon microscope coupled to a Luca R EMCCD camera or a FV1000 Olympus confocal microscope.

### Cortical granule staining

After live imaging the zona pellucida was removed by brief incubation in Tyrode's solution pH 2.2 and cells were fixed during 40 min in 3.7% paraformaldehyde in DPBS. They were washed in blocking solution (3 mg/ml BSA, 100 mM glycine and 0.01% Tween 20 in DPBS) during 15 min and then permeabilized with 0.1% Triton-X in DPBS for 15 min. Then, remanent cortical granules were stained by incubation with 25 µg/ml LCA-rhodamine (Vector Laboratories, Burlingame, CA) in blocking solution during 30 min. Cells were washed again in blocking solution during 15 min and finally were mounted using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). For 3D reconstruction, cells were mounted, without being smashed, in a chamber with solid vaseline around them.

### Image analysis

Cortical granules density per 100 $\mu m^2$ for each cell was determined from confocal images as the mean of the counts from at least four non overlapping equal areas of cortex containing cortical granules, according to our previous work (de Paola et al., 2015). Cortical granules density per 100 $\mu m^2$ was calculated by computer-assisted image quantification using ImageJ. Exudate dots were quantified in the same way, using the same areas selected for cortical granules quantification. 3D reconstruction was performed using '3D Project' plug in from

ImageJ (brightest point method) using single 2D confocal images taken every 2 µm along the z-axis. Surface plot was realized using the 'Surface Plot' command of ImageJ (polygon multiplier 100%). Kinetic of cortical reaction was measured from confocal images as fluorescent intensity (F) relative to baseline ($F_0$) using the formula: $F/F_0$-1, with the program ImageJ.

**Data analysis**

Experiments were repeated at least three times. Data analysis was performed using KyPlot or Statgraphics software. The percentage of responding oocytes in each condition was compared using the test of difference between two proportions [14]. Statistical significance of cortical granule density and exudate dot density was determined by Student's test, or Steel-Dwass test. The results are expressed as mean ± SD.$P < 0.05$ was considered statistically significant.


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# Competing interests

No competing interests declared


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## Reference List


Bello, O. D., Cappa, A. I., de, P. M., Zanetti, M. N., Fukuda, M., Fissore, R. A., Mayorga, L. S. and Michaut, M. A. (2016). Rab3A, a possible marker of cortical granules, participates in cortical granule exocytosis in mouse eggs. *Exp. Cell Res.* 347, 42-51.

Cheeseman, L. P., Boulanger, J., Bond, L. M. and Schuh, M. (2016). Two pathways regulate cortical granule translocation to prevent polyspermy in mouse oocytes. *Nat. Commun.* 7, 13726.

Cherr, G. N., Drobnis, E. Z. and Katz, D. F. (1988). Localization of cortical granule constituents before and after exocytosis in the hamster egg. *J. Exp. Zool.* 246, 81-93.

Connors, S. A., Kanatsu-Shinohara, M., Schultz, R. M. and Kopf, G. S. (1998). Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation, and cortical granule anchoring in mouse eggs. *Dev. Biol.* 200, 103-115.

Damiani, P., Fissore, R. A., Cibelli, J. B., Long, C. R., Balise, J. J., Robl, J. M. and Duby, R. T. (1996). Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. *Mol. Reprod. Dev.* 45, 521-534.

de Paola, M., Bello, O. D. and Michaut, M. A. (2015). Cortical Granule Exocytosis Is Mediated by Alpha-SNAP and N-Ethilmaleimide Sensitive Factor in Mouse Oocytes. *PLoS. One.* 10, e0135679.

Ducibella, T., Anderson, E., Albertini, D. F., Aalberg, J. and Rangarajan, S. (1988a). Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation. *Dev. Biol.* 130, 184-197.

Ducibella, T. and Buetow, J. (1994). Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. *Dev. Biol.* 165, 95-104.

Ducibella, T., Duffy, P. and Buetow, J. (1994). Quantification and localization of cortical granules during oogenesis in the mouse. *Biol. Reprod.* 50, 467-473.

Ducibella, T., Rangarajan, S. and Anderson, E. (1988b). The development of mouse oocyte cortical reaction competence is accompanied by major changes in cortical vesicles and not cortical granule depth. *Dev. Biol.* 130, 789-792.

Dumollard, R., Duchen, M. and Sardet, C. (2006). Calcium signals and mitochondria at fertilisation. *Semin. Cell Dev. Biol.* 17, 314-323.

Ikebuchi, Y., Masumoto, N., Matsuoka, T., Yokoi, T., Tahara, M., Tasaka, K., Miyake, A. and Murata, Y. (1998). SNAP-25 is essential for cortical granule exocytosis in mouse eggs. *Am. J. Physiol* 274, C1496-C1500.

Iwahashi, K., Kuji, N., Fujiwara, T., Tanaka, H., Takahashi, J., Inagaki, N., Komatsu, S., Yamamoto, A., Yoshimura, Y. and Akagawa, K. (2003). Expression of the exocytotic protein syntaxin in mouse oocytes. *Reproduction.* 126, 73-81.

**Kim, B., Zhang, X., Kan, R., Cohen, R., Mukai, C., Travis, A. J. and Coonrod, S. A. (2014). The role of MATER in endoplasmic reticulum distribution and calcium homeostasis in mouse oocytes.** ***Dev. Biol.*** **386, 331-339.**

**Latham, K. E. (2015). Endoplasmic reticulum stress signaling in mammalian oocytes and embryos: life in balance.** ***Int. Rev. Cell Mol. Biol.*** **316, 227-265.**

**Li, R. and Albertini, D. F. (2013). The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte.** ***Nat. Rev. Mol. Cell Biol.*** **14, 141-152.**

**Liu, M. (2011). The biology and dynamics of mammalian cortical granules.** ***Reprod. Biol. Endocrinol.*** **9, 149.**

**Reader, K. L., Stanton, J. L. and Juengel, J. L. (2017). The Role of Oocyte Organelles in Determining Developmental Competence.** ***Biology. (Basel)*** **6.**

**Satouh, Y., Nozawa, K., Yamagata, K., Fujimoto, T. and Ikawa, M. (2017). Viable offspring after imaging of Ca2+ oscillations and visualization of the cortical reaction in mouse eggs.** ***Biol. Reprod.*** **96, 563-575.**

**Sun, Q. Y. (2003). Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs.** ***Microsc. Res. Tech.*** **61, 342-348.**

**Tsai, P. S., van, H. T. and Gadella, B. M. (2011). Preparation of the cortical reaction: maturation-dependent migration of SNARE proteins, clathrin, and complexin to the porcine oocyte's surface blocks membrane traffic until fertilization.** ***Biol. Reprod.*** **84, 327-335.**

**Zarski, D., Krejszeff, S., Palinska, K., Targonska, K., Kupren, K., Fontaine, P., Kestemont, P. and Kucharczyk, D. (2012). Cortical reaction as an egg quality indicator in artificial reproduction of pikeperch, Sander lucioperca.** ***Reprod. Fertil. Dev.*** **24, 843-850.**

## Figures

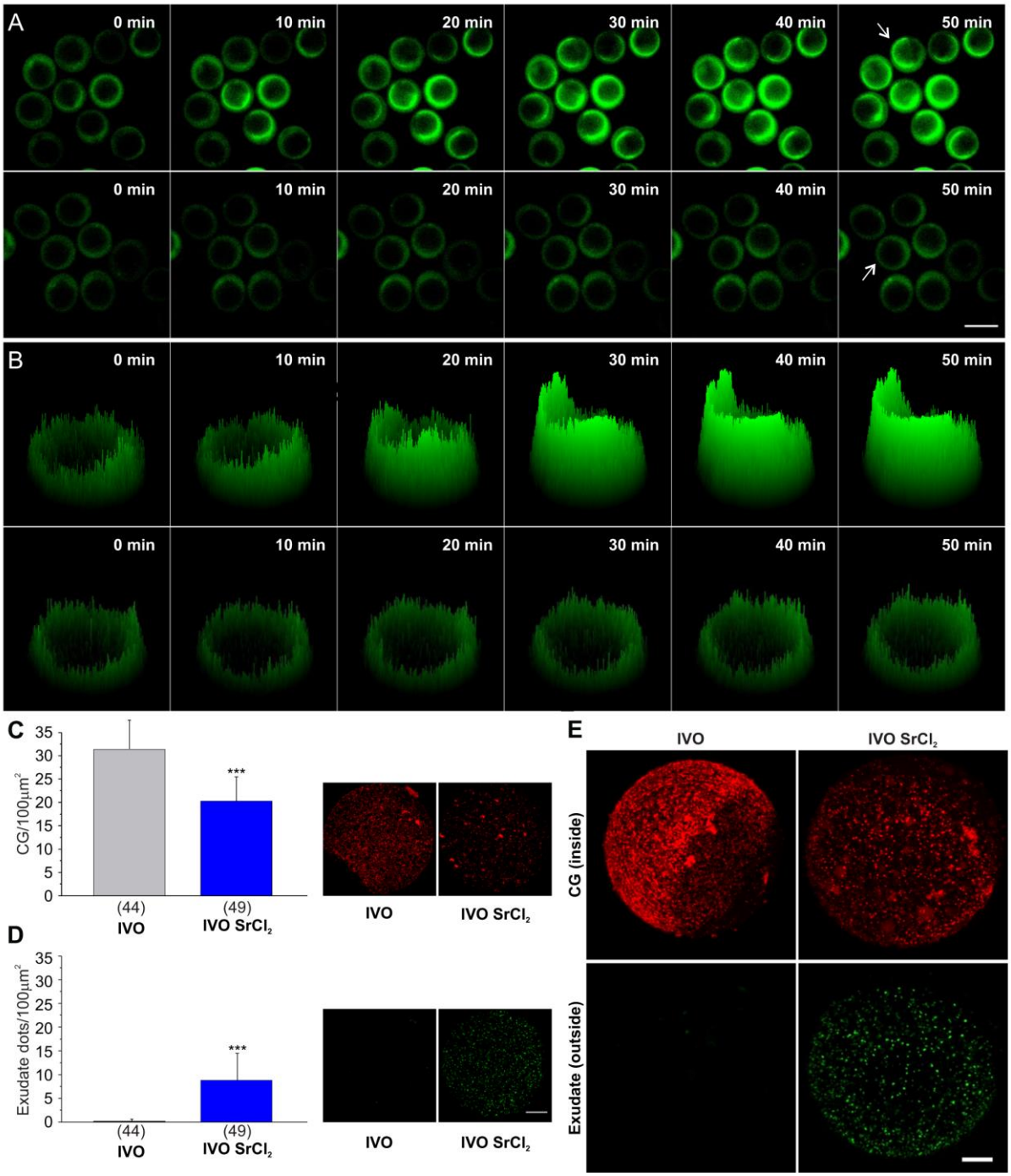


**Fig 1**. **Cortical reaction in real time**. **A.** Epifluorescence microscopy images of *in vivo* ovulated (IVO) oocytes population incubated with (up) or without (down) $SrCl_2$ in presence of LCA-FITC. Scale bar: 100 µm. **B.** 3D Surface plot of the oocyte indicated by arrow in A: oocyte incubated with (up) or without (down) $SrCl_2$. **C.** Cortical granules density per 100 $\mu m^2$ (CG/100 $\mu m^2$) in IVO oocytes activated (blue bar) or not activated (gray bar) with $SrCl_2$. On the right side of bars, representative confocal images of cortical granules are shown. **D.** Exudate dots density per 100 $\mu m^2$ and representative confocal images of exudate from the same oocytes evaluated in C. Data in C and D are shown as mean ± SD from at least 3 independent experiments. Numbers in parentheses represent total number of oocytes. Different letters indicate statistically significant differences between groups ($p<0.05$, Student's test). Scale bar: 20 µm. **E.** 3D reconstruction from multiple confocal images of two representative IVO oocytes showing cortical granules (up) and exudate (down) in the presence (right) or absence (left) of $SrCl_2$. Red color (LCA-rhodamine) shows cortical granules that remain in the cell (CG inside). Green color (LCA-FITC) shows exudate´s dots produced by the release of cortical granules content in presence of LCA-FITC after cell fixation (Exudate outside). Images of IVO condition correspond to the same oocyte; the same, for IVO $SrCl_2$. Scale bar: 10 µm.

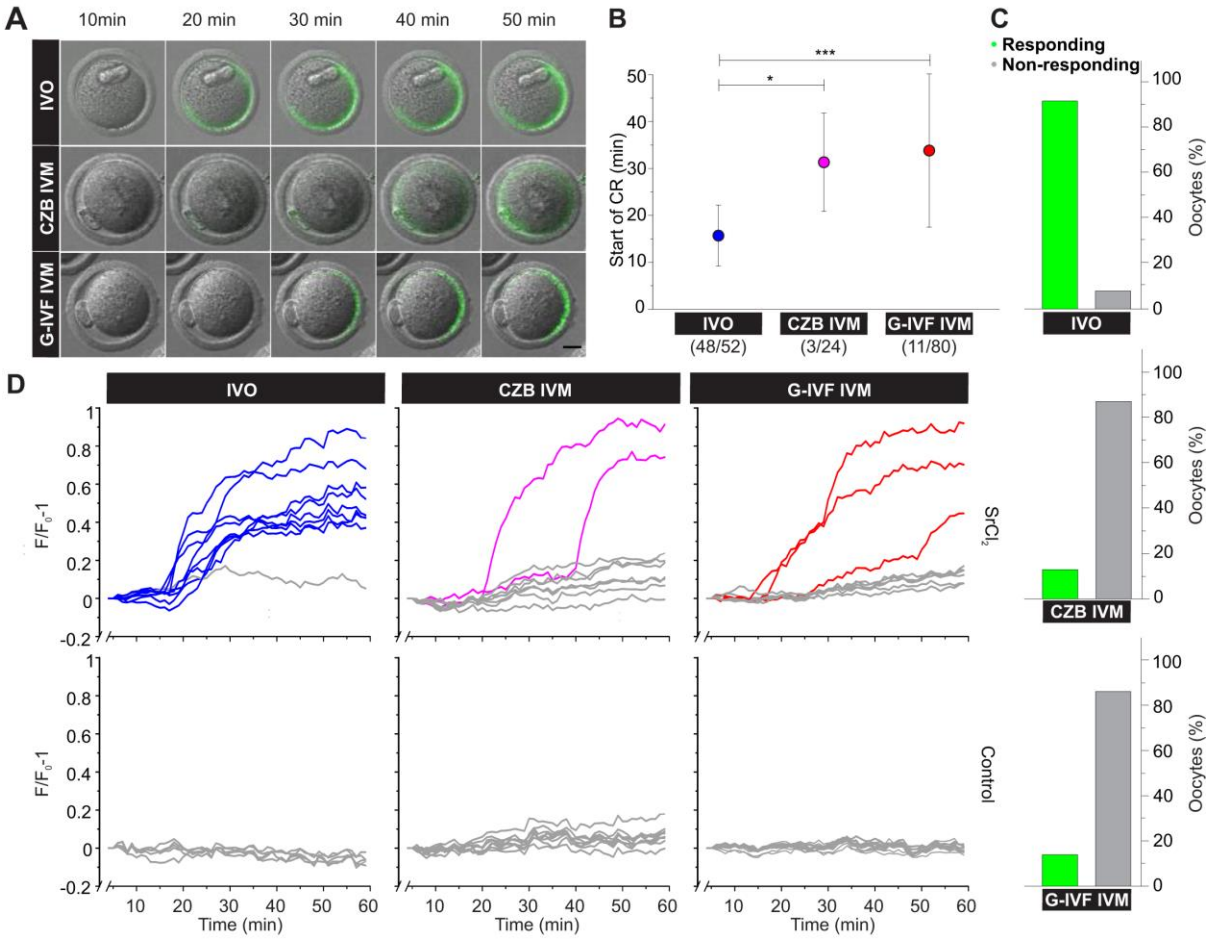



**Fig 2. Live imaging of cortical reaction in ovulated oocytes (IVO) and *in vitro* matured (IVM) oocytes. A.** Merged images from FV1000 Olympus confocal microscope from DIC and FITC channels showing a representative $SrCl_2$-activated oocyte in presence of LCA-FITC for IVO, CZB IVM and G-IVF IVM oocytes. Scale bar: 20 µm. **B.** Start time of cortical reaction (CR) represented as mean ± SD from at least 3 independent experiments. Numbers in parentheses represent number of responding oocytes from total. Different letters indicate statistically significant differences between groups ($p<0.05$, Tukey's test). **C.** Percentage of responding (green bar) and non-responding oocytes (grey bar) under $SrCl_2$ stimulation in the same experiments evaluated in B. Top to bottom: IVO (n= 52), CZB IVM (n= 24) and G-IVF IVM (n= 80) oocytes. Percentages of IVM responding oocytes (green bars) were significatively different compared to IVO responding oocytes ($p<0.0001$, test of difference between two proportions). **D.** Kinetic of cortical reaction. Fluorescence´s increment represented as ($F/F_0$-1) versus time from IVO, CZB IVM and G-IVF IVM oocytes incubated with (up) or without (down) $SrCl_2$ during 1 h in presence of LCA-FITC. Gray lines represent non responding oocytes and color lines represent responding oocytes.

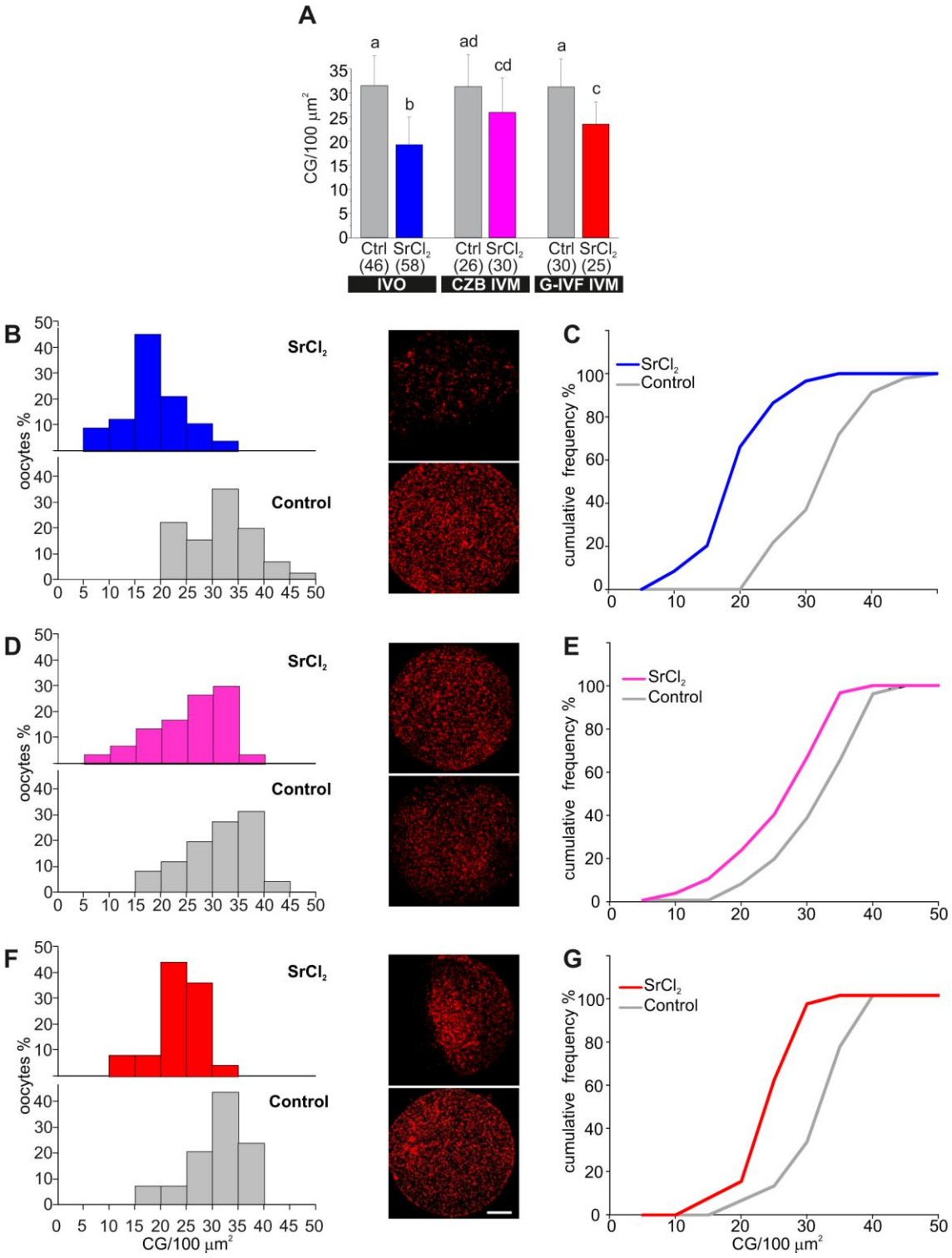



**Fig 3. Quantification of cortical granule (CG) exocytosis in fixed oocytes.A.** Bar graph of cortical granules density (CG/100 $\mu m^2$) of treated oocytes with ($SrCl_2$) or without $SrCl_2$ (Ctrl) for each condition: *in vivo* ovulated (IVO), CZB *in vitro* matured (CZB IVM) and G-IVF *in vitro* matured (G-IVF IVM) oocytes. Data are shown as mean ± SD from at least 3

independent experiments; numbers in parentheses represent the total number of evaluated oocytes. Different letters indicate statistically significant differences between groups ($p<0.05$, Steel-Dwass test). **B**, **D** and **F**: relative frequency histograms of cortical granules density for the same oocytes analyzed in A. Representative confocal images of oocytes subjected to each treatment are shown on the right. Cortical granules were labeled with LCA-rhodamine. Scale bar: 20 μm.**C, E** and **G**: cumulative relative frequency of cortical granules density for the same oocytes analyzed in A.

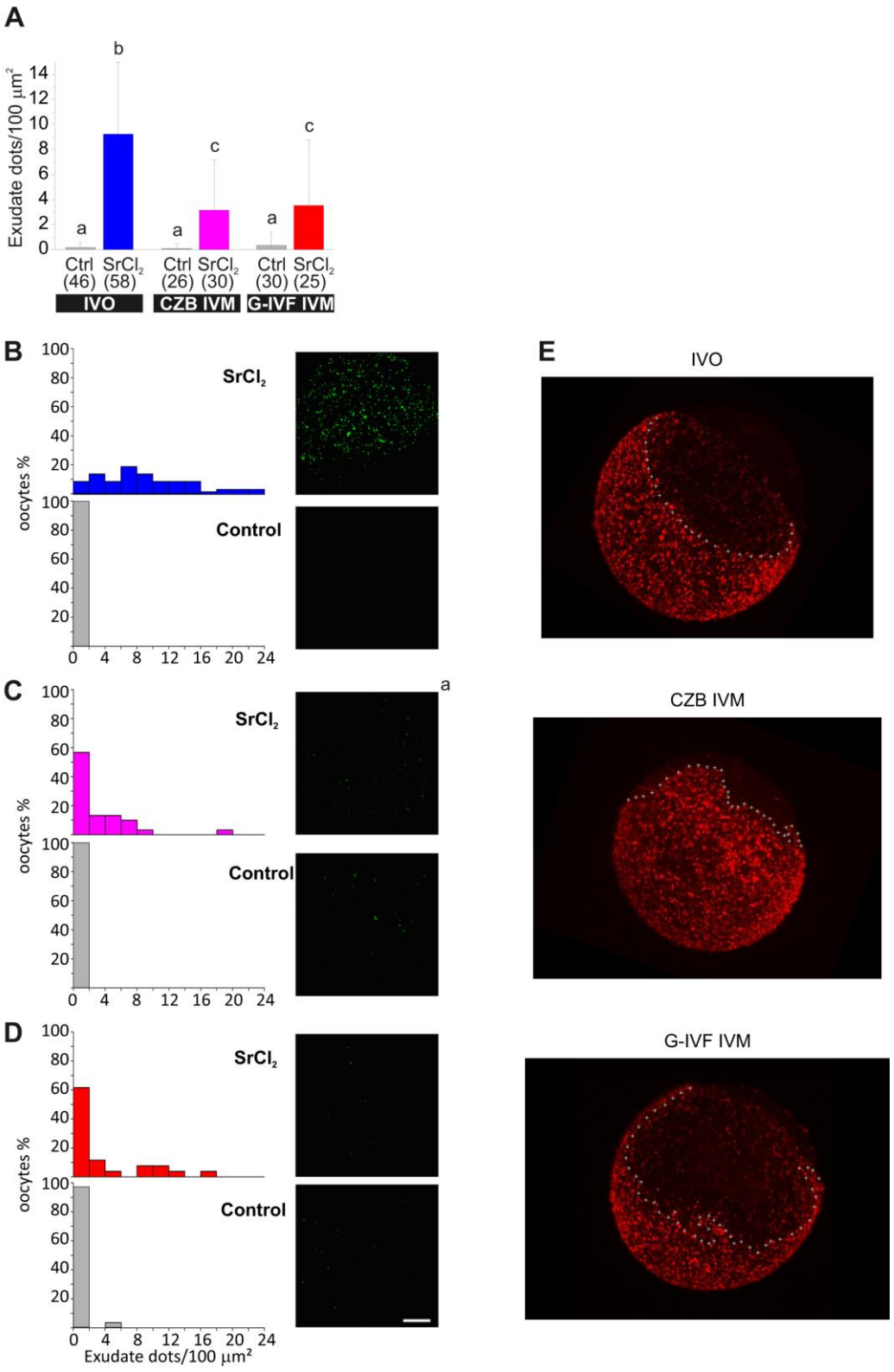



**Fig 4.Quantification of exudate dots in fixed oocytes and cortical granule localization. A.** Bar graph of exudate dots density per 100 µm$^2$ of oocytes analyzed in Fig. 3. Data are shown as mean ± SD from at least 3 independent experiments; numbers in parentheses represent the total number of evaluated oocytes. Different letters indicate statistically significant

differences between groups ($p<0.05$, Steel-Dwass test). **B**, **C** and **D**: relative frequency histograms of exudate dots density for the same oocytes analyzed in A. Representative confocal images of the same oocytes from Fig. 3 are shown on the right, now showing exudate labeled with LCA-FITC. Scale bar: 20 μm. **E:** Distribution of cortical granules. 3D reconstruction from multiple confocal images of representative oocytes showing cortical granules distribution labeled with LCA-rhodamine in ovulated and *in vitro* matured oocytes. IVO, ovulated oocyte; CZB IVM, CZB *in vitro* matured oocyte; G-IVF IVM, G-IVF *in vitro* matured oocyte.

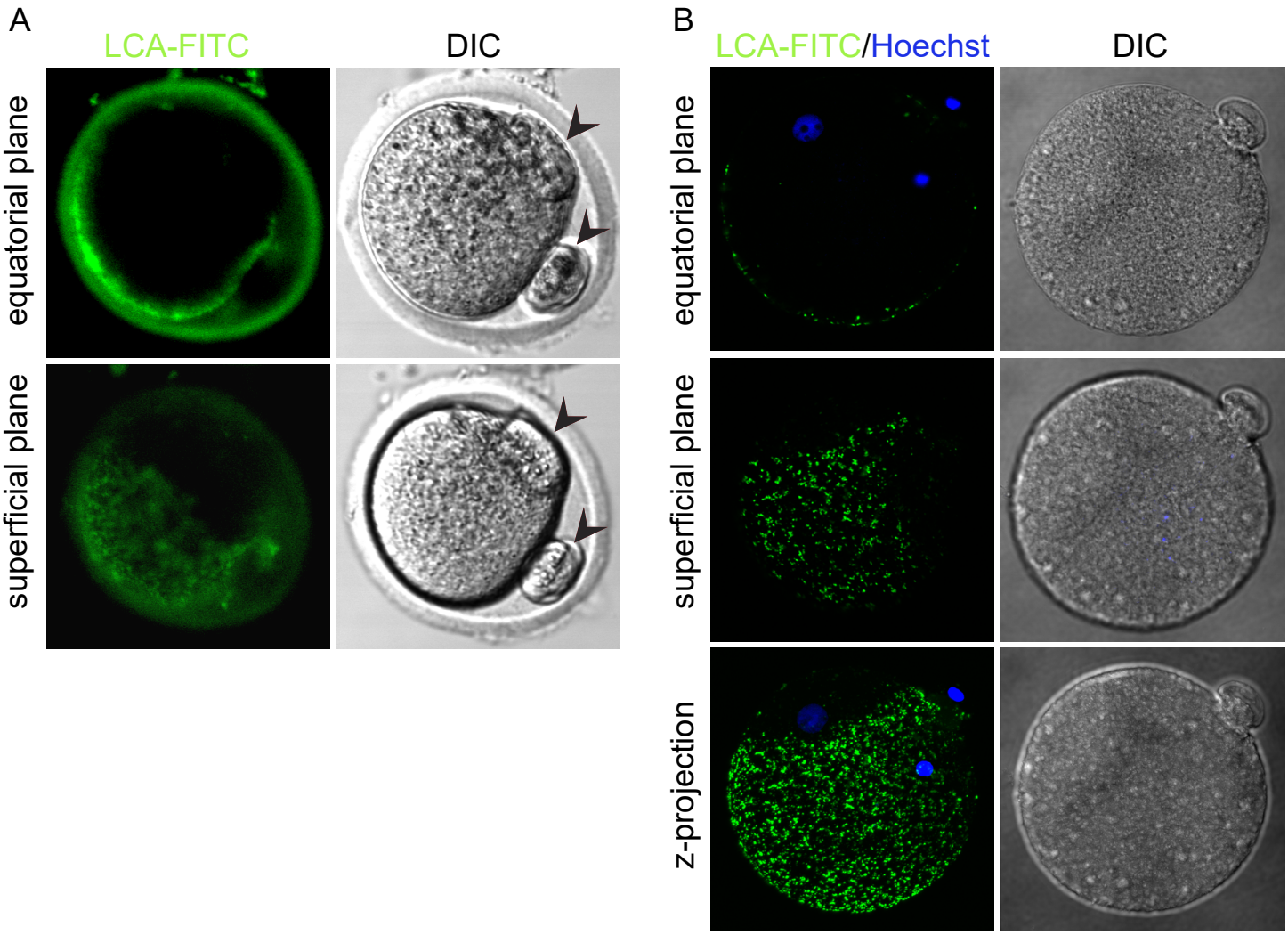
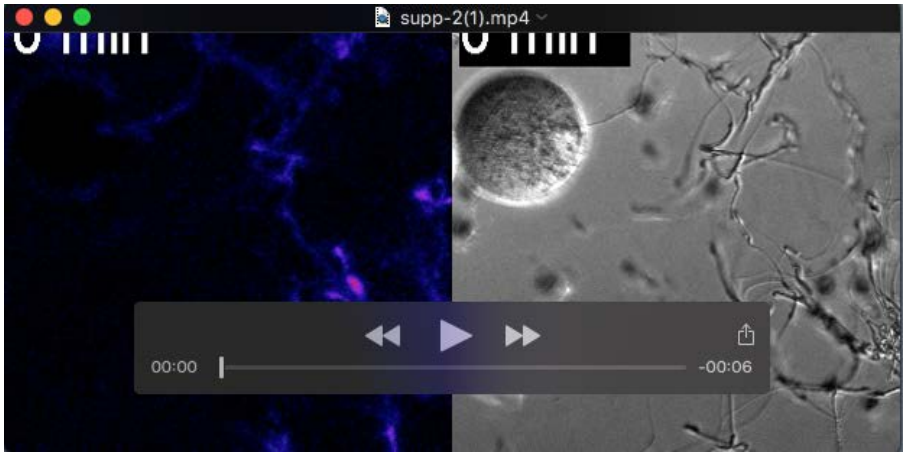
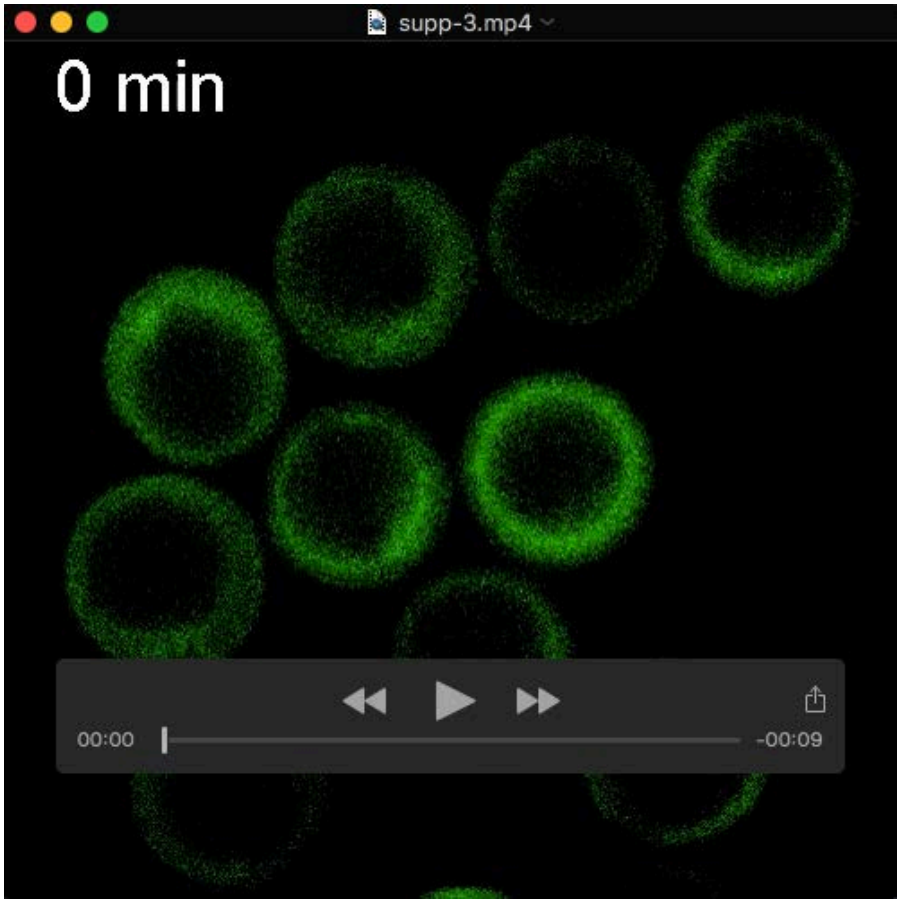


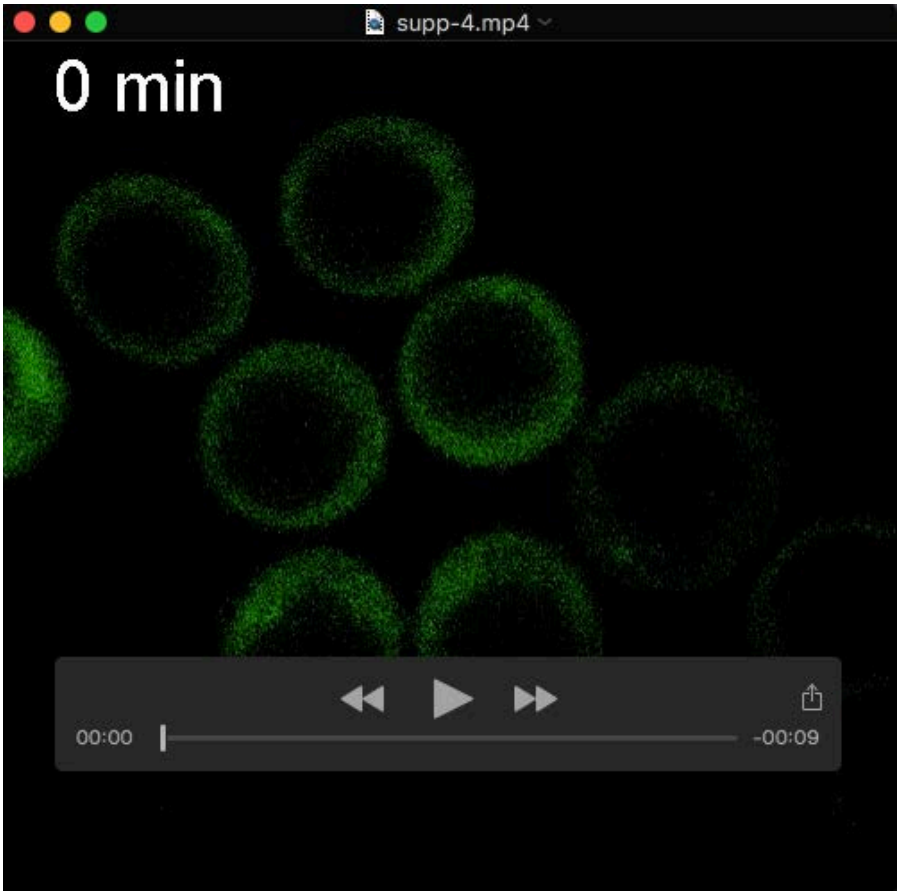

**Figure S1. *In vitro* fertilized eggs stained with LCA-FITC.** Eggs were inseminated with capacitated spermatozoa at a final concentration of 1-5 × $10^6$ per ml for 2 hours. After washing, embryos were incubated in CZB medium supplemented with 5 µg/ml LCA–FITC for additional 5 hours. A. 1-cell embryo stained with LCA-FITC (live cell, confocal images); arrow heads indicate polar bodies. B. 1 cell embryo stained with LCA-FITC after fixation (fixed cell, confocal images and Z-projection of 3D reconstruction). Blue, DNA stained with Hoechst 3342. DIC, differential interference contrast.



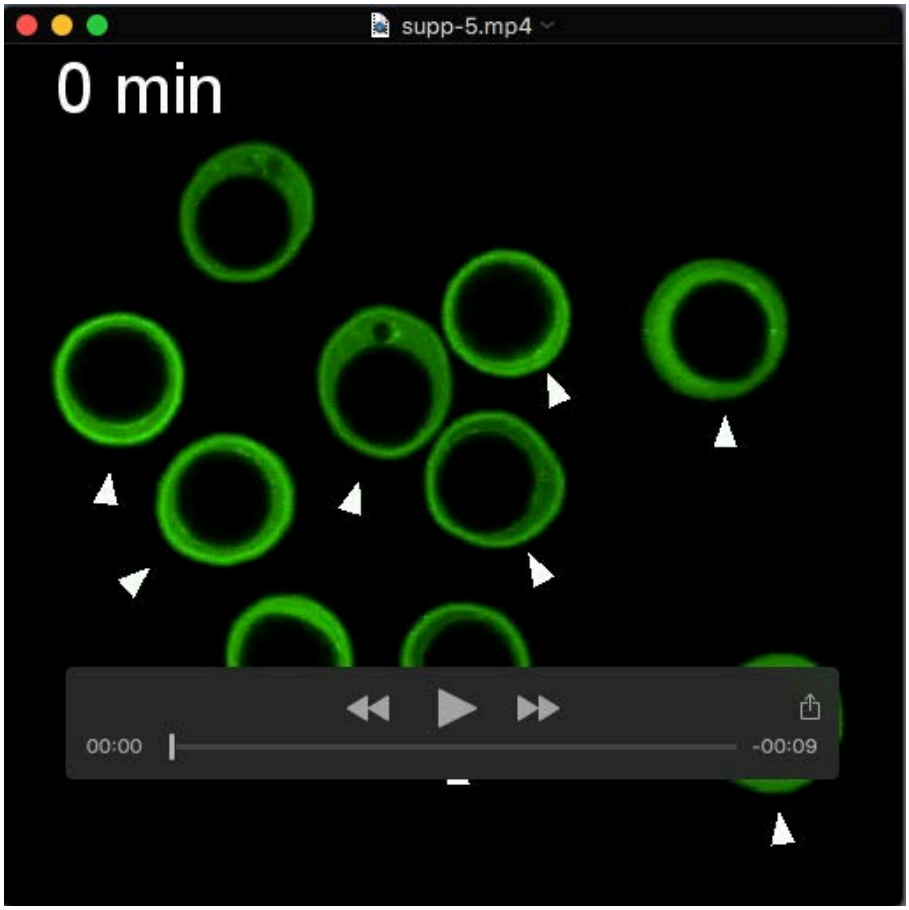

**Movie 1. In vitro fertilization in presence of LCA-FITC:** Confocal time lapse (left) and Differential interference contrast (DIC, right) microscopy of IVO oocytes incubated with mouse sperm in presence of fluorescenc lectin LCA-FITC.



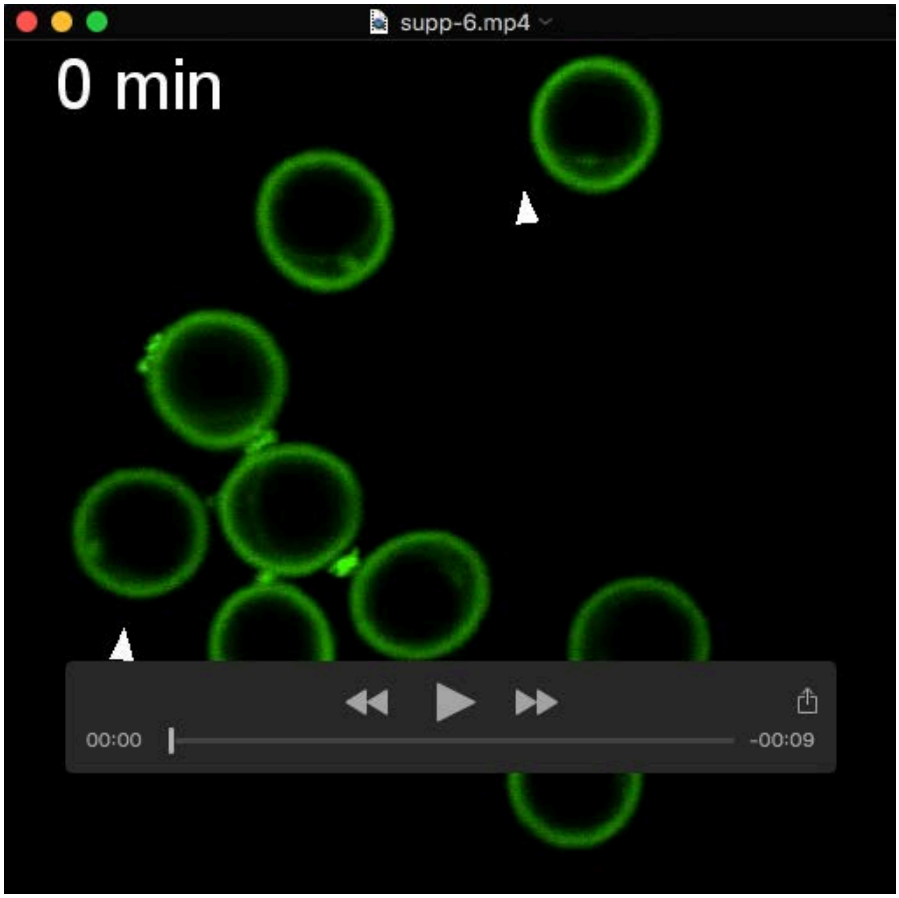

**Movie 2. Live imaging of cortical reaction in ovulated oocytes: experimental condition.** Epifluorescence time lapse of *in vivo* matured oocytes incubated with $SrCl_2$ in presence of fluorescent lectin LCA-FITC.



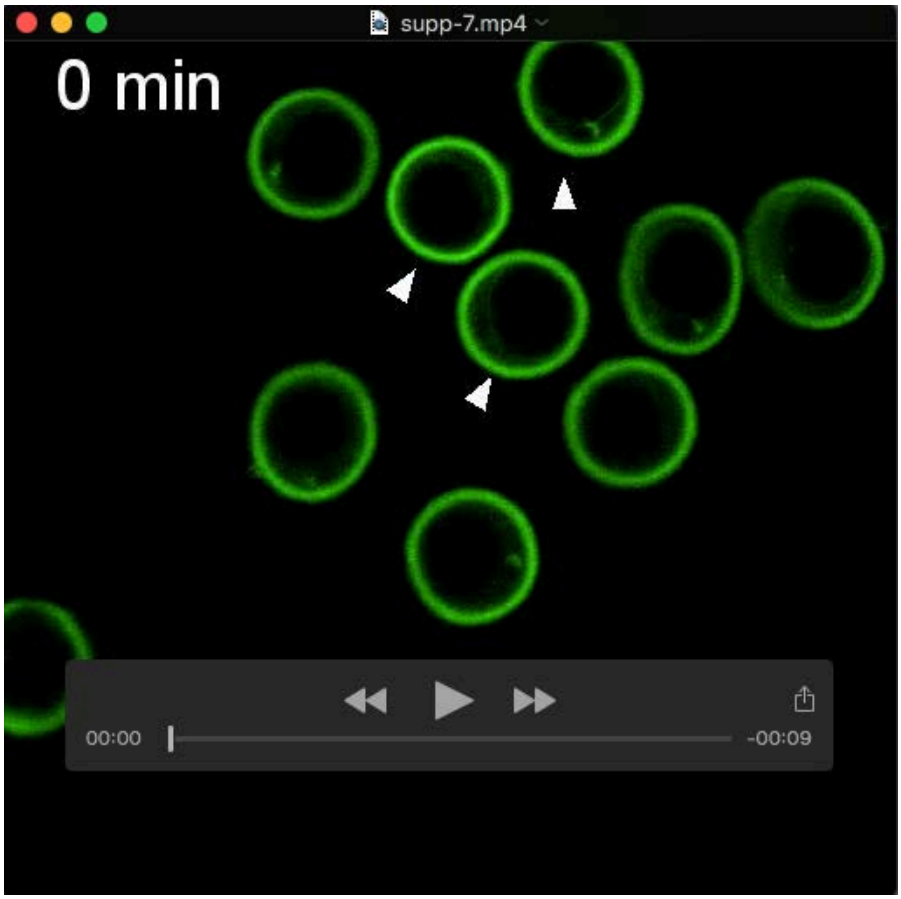

**Movie 3. Live imaging of cortical reaction in ovulated oocytes: control condition.**
Epifluorescence time lapse of *in vivo* matured oocytes incubated without $SrCl_2$ in presence of LCA-FITC.




**Movie 4. Live imaging of cortical reaction in ovulated oocytes (IVO).** Confocal time lapse of IVO oocytes incubated with $SrCl_2$ and LCA-FITC. Arrowheads indicate responding cells.




**Movie 5. Live imaging of cortical reaction in CZB *in vitro* matured oocytes (CZB IVM).** Confocal time lapse of CZB IVM oocytes incubated with $SrCl_2$ and LCA-FITC. Arrowheads indicate responding cells.




**Movie 6. Live imaging of cortical reaction in G-IVF *in vitro* matured oocytes (G-IVF IVM).** Confocal time lapse of G-IVF IVM oocytes incubated with $SrCl_2$ and LCA-FITC. Arrowheads indicate responding cells.