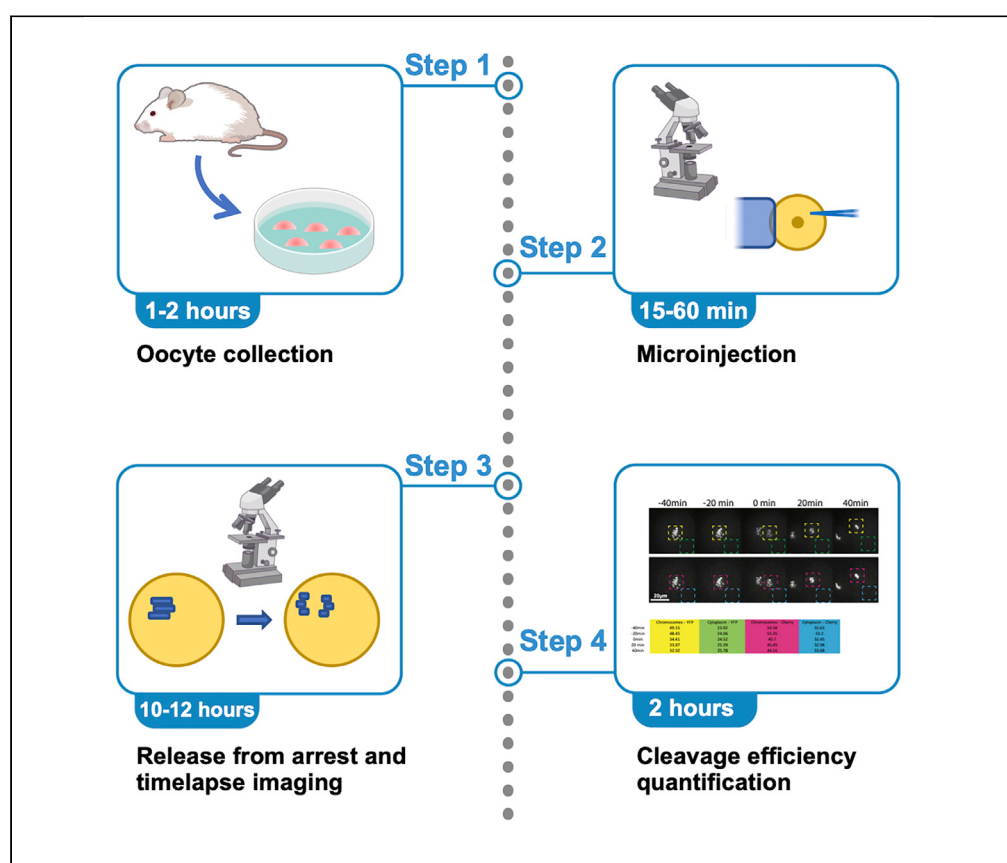


Protocol

Protocol to measure cleavage efficiency of the meiotic cohesin subunit Rec8 by separase in mouse oocytes using a biosensor



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Highlights

Mouse oocyte
collection and
microinjection

Timelapse imaging of
mouse oocyte
maturation

Biosensor to measure
efficiency of Rec8
cleavage by separase

Here, we describe a biosensor to assess meiotic cohesin subunit Rec8 cleavage in mouse oocytes. We detail oocyte collection and microinjection of the mRNA expressing the biosensor. The biosensor is targeted to chromosomes and consists of two fluorophores flanking a Rec8 fragment containing separase cleavage sites. Cleavage leads to dissociation of one fluorophore from chromosomes, and the efficiency can be estimated by live imaging. We detail the use of this biosensor in mouse oocytes with or without Aurora B/C inhibitor.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

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SUMMARY

Here, we describe a biosensor to assess meiotic cohesin subunit Rec8 cleavage in mouse oocytes. We detail oocyte collection and microinjection of the mRNA expressing the biosensor. The biosensor is targeted to chromosomes and consists of two fluorophores flanking a Rec8 fragment containing separase cleavage sites. Cleavage leads to dissociation of one fluorophore from chromosomes, and the efficiency can be estimated by live imaging. We detail the use of this biosensor in mouse oocytes with or without Aurora B/C inhibitor. For complete details on the use and execution of this protocol, please refer to Nikalayevich et al. (2022).

BEFORE YOU BEGIN

⌚ Timing: 1–4 weeks for oocyte culture optimization, 1 week for sequence verification and preparing capped mRNA

This protocol describes assessment of cleavage efficiency of a biosensor containing a Rec8 fragment, in wild type mouse oocytes (Swiss) completing the first meiotic division, in presence or absence of Aurora B/C inhibitors. We also used this biosensor in Separase conditional knockout oocytes with a C57BL6 genetic background. This protocol is based on (Nikalayevich et al., 2018) where a biosensor containing a fragment of the mitotic cohesin subunit Scc1 was used to detect Separase activity.

1. Make sure to have the necessary permission and training to euthanize mice.
2. Oocyte culture conditions during timelapse imaging should be optimal to let oocytes go through anaphase without delay. See [problem 1](#) in the [troubleshooting](#) section.
3. Verify the design of the biosensor, making sure it contains Separase cleavage sites (ExxR/D) only where you intend them to be.

Note: Artificial cleavage sites can be introduced inadvertently during the cloning process, please check the peptide sequence of any linkers between the functional parts of the biosensor.



4. Prepare capped mRNA following the [instructions](#) of the kit's manufacturer. Aliquot the final elution (30–40 μ L) in 5 μ L aliquots, store at -20°C for up to 1 month (or at -80°C for longer periods).

△ CRITICAL: Use gloves, work in a clean and nuclease-free environment (preferentially with RNAse inhibitors) and use nuclease-free water when preparing mRNA.

Note: The level of biosensor expression depends on the quality of the microinjected mRNA. The mRNA should form two sharp bands on the agarose gel after electrophoresis, appearance of a smear signifies RNA degradation. If the biosensor sequence is rather long (above 2,500 bps), additional polyadenylation and increasing the concentration of GTP in the *in vitro* transcription reaction such as suggested by the manufacturer can help with increasing mRNA yield and stability, leading to higher protein expression in oocytes.

Institutional permissions

Any experiments on live vertebrates must be performed in accordance to your institutional and national guidelines. Please acquire necessary permissions before performing this protocol.

To perform rodent animal experiments in France, French government-issued authorization is required for the animal facility and for the user. The project also has to be reviewed by a local ethics committee.

For the experiments described here, the authorization B75-05-13 was applied for the animal facility, and the Charles Darwin (Nr. 5, Ile-de-France) ethical committee authorized the project to be implemented by the users with the required and verified skills in animal handling.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
M2 medium	Merck Millipore	Cat# MR-015P
EmbryoMax® Penicillin/Streptomycin, solution 100×	Merck Millipore	Cat# TMS-AB2-C
N ₆ ,2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (dbcAmp)	Sigma-Aldrich	Cat# D0260-25MG CAS Number: 16980-89-5
Mineral Oil	Sigma-Aldrich	Cat# M5310 CAS Number: 8042-47-5
ZM447439	Tocris	Cat# 2458 CAS Number: 331771-20-1
AZD1152	Sigma-Aldrich	SML0268 CAS Number: 722544-51-6
MLN 8237	Bertin Bioreagent	Cat# 13602 CAS Number: 1028486-01-2
Critical commercial assays		
mMESSAGE mACHINE T3 Kit	Life Technologies (Ambion)	Cat# AM1348
RNeasy Mini Kit	QIAGEN	Cat# 74104
Experimental models: Organisms/strains		
Mouse: SWISS CD-1, 8–12 weeks old females	JANVIER LABS	RjOri:SWISS
Recombinant DNA		
pRN3-H2B-mCherry-Rec8(380-486aa)-YFP	Nikalayevich et al. (2022)	NA
Software and algorithms		
ImageJ	N/A	https://imagej.nih.gov/ij/
Graphpad Prism 9	GraphPad software	https://www.graphpad.com/scientific-software/prism/
MS Excel	Microsoft	https://www.microsoft.com/

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Corning™ Falcon™ Easy-Grip Tissue Culture Dishes 60 mm	Fisher Scientific	Cat# 10099170
Corning™ Falcon™ Easy-Grip Tissue Culture Dishes 30 mm	Fisher Scientific	Cat# 10038820
Stereomicroscope	Nikon	SMZ1000
Thermoplate	TOKAI HIT	NA
Parafilm M	Sigma-Aldrich	#P7543
Aspirator tube assemblies for calibrated microcapillary pipettes product	Sigma-Aldrich	#A5177-5EA
Fisherbrand™ Translucent Platinum-Cured Silicone Tubing	Fisher Scientific	#11748118 #11758148
230 mm soda-lime glass Pasteur pipettes with a closed tip	CEB	#PP230S
STRIPPER pipetter	CooperSurgical	#MXL3-STR
Pre-manufactured tips with 200 µm inner diameter	CooperSurgical	#MXL3-200
Pre-manufactured tips with 125 µm inner diameter	CooperSurgical	#MXL3-125
Glass capillaries	Harvard Apparatus	#W3 30-0038
Puller apparatus	NARISHIGE	Narishige PC-100
TransferMan® 4r micromanipulators	Eppendorf	#5193000012
Electronic microinjector FemtoJet 4i	Eppendorf	#5252000013
Manual microinjector CellTram 4r Oil	Eppendorf	# 5196000030
Cavity Well Microscope Slides for Cell Cultures and Liquids, Single Cavity, 25 × 75 mm	Globe Scientific	#1341-72
Glass Depression Slides	VWR	# 470235-728
VacuTipl microcapillaries	Eppendorf	#5195000036
35 mm Dish Type 1-Well Magnetic Chamber for Round Coverslips	Live Cell Instrument	#CM-XX-X
FluoroDish Cell Culture Dish - 35 mm	World Precision Instruments	#FD35-100

MATERIALS AND EQUIPMENT

For oocyte culture we use Corning™ Falcon™ Easy-Grip Tissue Culture Dishes with a diameter of 60 mm (Fisher Scientific ref. #10099170) or 30 mm (Fisher Scientific ref. #10038820).

The culture medium (M2 medium) is supplemented with penicillin (final concentration 1.678 mM) and streptomycin (final concentration 0.689 mM). The supplemented medium can be stored at 4°C for 2–4 weeks in 3 mL aliquots. To keep the oocytes in prophase I arrest, the medium is additionally supplemented with 100 µg/mL dibutyl cyclic AMP (dbcAMP). The medium supplemented with dbcAMP should be used within 12 h.

Penicillin/Streptomycin-supplemented M2 medium

Reagent	Stock concentration	Final concentration	Add to 100 mL
M2 medium			99 mL
EmbryoMax® Penicillin/Streptomycin, solution 100×	10,000 Units Penicillin and 10,000 µg Streptomycin per mL	100 Units Penicillin and 100 µg Streptomycin per mL	1 mL

dbcAMP-supplemented M2 medium

Reagent	Stock concentration	Final concentration	Add to 1 mL
Penicillin/Streptomycin-supplemented M2 medium			999 µL
dbcAMP	100 mg/mL	100 µg/mL	1 µL

Oocyte collection and manipulation is done under a Nikon stereomicroscope SMZ1000 equipped with a compatible Tokai Hit thermoplate. The light source should be below the stage, and the light temperature should be warm (i.e., the color of the light should be slightly yellow) for better visualization of the oocytes.

Silicone tubing for mouth pipetting consists of a mouth piece attached to a thin (4.8 mm inner diameter) silicon tube of about 1 m. On the other side, a 1 mL plastic pipette tip is introduced and fixed with the help of Parafilm M (Sigma-Aldrich, ref. #P7543). A thick (1 mm inner diameter), around 5 cm long silicon tube is pulled over the larger end of the 1 mL tip and again sealed with Parafilm. This side is used to attach the thicker end of a glass Pasteur pipette.

The mouth piece is taken from the “Aspirator tube assemblies for calibrated microcapillary pipettes” product (Sigma-Aldrich ref. #A5177-5EA). Alternatively, the mouth piece can be replaced with a 200 μ L plastic pipette tip, with its tapered end inserted into the silicon tube. The silicon tubing is Fisherbrand™ Translucent Platinum-Cured Silicone Tubing (Fisher Scientific ref. #11748118 and #11758148).

We use 230 mm Pasteur pipettes with a closed tip made from soda-lime glass (CEB ref. #PP230S) to generate very thin glass pipette tips for oocyte aspiration. The thin part of the pipette (around 1–2 cm away from the part where the pipette gets wide) should be heated up over a flame until it is red and glowing. Holding both ends of the Pasteur pipette, the molded glass should be extended with a swift motion outside the flame. The very end of the extended glass pipette should be broken off manually to produce a 5–10 cm long thin ending with an opening large enough to aspirate oocytes. For oocyte collection, the inner diameter of the opening should be large enough to aspirate oocytes with attached cumulus cells (around 200 μ m). For subsequent cleaning of oocytes, the inner diameter of the pipette should be very close to the diameter of the oocytes (70–80 μ m). The correct size of the pipette tip has to be checked under the microscope and by mouth-pipetting oocytes up and down.

Alternative to mouth pipetting: STRIPPER pipetters (CooperSurgical ref. #MXL3-STR) and pre-manufactured tips with different inner diameters: 200 μ m for manipulation (Cooper Surgical, ref. #MXL3-200) and 125 μ m for denudation (CooperSurgical, ref. #MXL3-125).

To manufacture the microinjection needles we use glass capillaries (Harvard apparatus, ref. #W3 30-0038) that were extended with the help of a Narishige puller (Narishige PN-31 used in this study is discontinued, Narishige PC-100 seems to be a suitable alternative).

The microinjection setup consists of an inverted light microscope (we use a Nikon Eclipse Ti) with a thermoplate (TOKAI HIT, the model has to be compatible with the microscope), and two TransferMan® 4r micromanipulators (Eppendorf, ref. #5193000012) that are used to control an electronic microinjector such as FemtoJet 4i (Eppendorf, ref. #5252000013), and a manual microinjector such as CellTram 4r Oil (Eppendorf, ref. #5196000030), which is used to maintain oocytes in position for injection. We use glass slides with a shallow depression (“depression glass slides” or “cavity glass slides”, for example Globe Scientific ref. #1341-72 or VWR ref. #470235-728) to place the oocytes under the microscope, and VacuTipl microcapillaries as holding pipettes (Eppendorf, ref. #5195000036).

For live imaging an inverted confocal microscope is needed, equipped with a spinning disc, an automated stage, camera (such as EMCCD with a pixel size of 16 $\times$ 16 μ m), 488 nm and 561 nm light sources, and transillumination. The microscope should be equipped with either an incubation chamber or a heated insert, for example PeCon (heating unit and an insert, compatible with the microscope). A 40 $\times$ objective with NA 1.4 or better and adapted for live imaging can be used.

For the microscopic imaging chamber, we use reusable chambers like the Chamlide chamber (Live Cell Instrument, ref. #CM-XX-X). Alternatively, single-use chambers like FluoroDish (World Precision Instruments, ref. #FD35-100) can be used.

STEP-BY-STEP METHOD DETAILS

Oocyte collection

⌚ Timing: 1–2 h

Mouse prophase-arrested oocytes are collected, cleaned and prepared for microinjection.

1. Prepare a 60 mm polystyrene tissue culture dish with 5–6 droplets (around 30 μ L each) of pre-warmed medium containing dbcAMP.
2. Cover the drops completely with a layer of mineral oil.
3. Prepare another dish/cover/container with a large drop of medium (800–1,000 μ L) containing dbcAMP and without oil for dissection of ovaries.
4. Sacrifice a 8–12 weeks old female mouse through cervical dislocation or in a CO₂ chamber, dissect the abdomen and collect the ovaries.

Note: Make sure to carefully tear off fatty tissue with forceps. You can lightly "roll" the collected ovaries on tissue paper to remove the remaining fatty tissue.

5. Place the ovaries in the dish prepared for dissection (with a large drop of medium (800–1,000 μ L) containing dbcAMP and without oil).

Note: We use mice that have not been hormonally stimulated, to work with naturally competent oocytes. Optimal age of a OF1 (Swiss) mouse for oocyte collection is 8–12 weeks. The quantity of oocytes obtained per mouse depends on the strain, the age and estrus cycle of the mouse. On average, an unstimulated OF1 mouse yields 40–70 oocytes, C57BL6 mouse yields around 30 oocytes.

6. Working under the binocular microscope equipped with a heated glass plate, macerate the ovaries with the sharp tip of a needle, opening up all the large follicles.
7. With a glass pipette (torn-out Pasteur pipettes, prepared as described above) collect all fully-grown oocytes that were released from the dissected follicles and transfer them to the dish with smaller medium droplets (around 30 μ L) covered with mineral oil.

Note: The end of the Pasteur pipette may be broken off manually to create a straight tip, to avoid hurting the oocytes upon pipetting. Oocytes should enter the glass pipette without extra sucking force applied and should not be deformed upon entering and exiting the glass pipette. The inner diameter should not be too large either, to avoid pipetting more volume than necessary at each step, which can make it hard to control the flow and leads to damage on the oocytes.

Note: In this and all following steps keep the oocytes at 37°C. This is important even when harvesting and manipulating oocytes. Medium should be prewarmed, and microscopes equipped with heated plates. Alternatively, oocytes can be put in an incubator between the different steps to avoid cooling down of the medium; however, in our experience proceeding like this is suboptimal and delays in meiotic maturation may occur.

8. Clean the collected oocytes. This means detaching all the remaining follicular cells (denudation) by passing the oocytes several times through the opening of a glass pipette with an inner diameter only slightly larger than the diameter of an oocyte (80–100 μ m).
9. Transfer the follicle-free oocytes into another medium drop in the same dish.
10. Select fully-grown competent oocytes that are arrested in prophase I, and discard the rest.

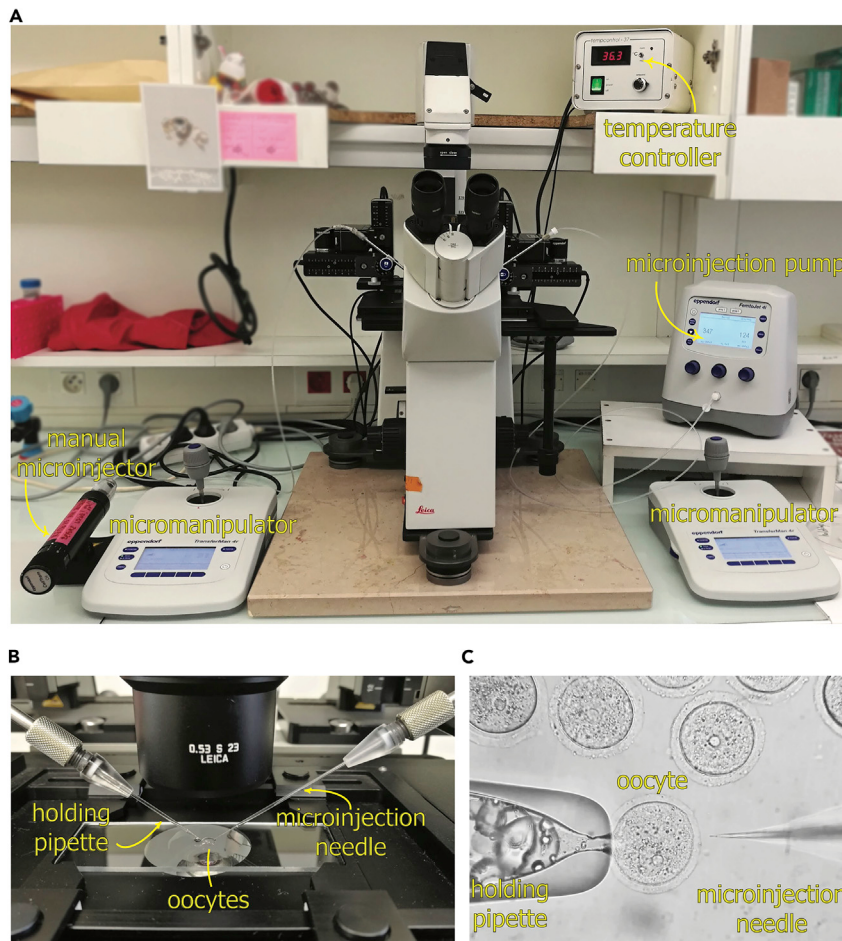


Figure 1. Microinjection setup

(A) A bright-field microscope with attached pipette holders that are operated by micromanipulators. Pressure is supplied by the manual microinjector to the left and the automatic microinjector to the right. The microscope is equipped with a heating plate and a temperature controller.
(B) A heating plate containing a depression glass slide. A holding pipette and a microinjection needle are connected to the pipette holders.
(C) Mouse oocyte ready for microinjection.

Note: Competent prophase I oocytes have reached their full size (70–80 μm in diameter), with the germinal vesicle (GV) not completely off-centered and with a smooth-looking Zona Pellucida (ZP). Oocytes covered with follicle cells that cannot be removed and the GV not in the center are not fully competent and should be discarded. See Figure 1C for an example of a competent oocyte arrested in prophase I.

Microinjection

⌚ Timing: 15–60 min (without centrifugation step)

mRNA to express the biosensor is microinjected into prophase-arrested oocytes.

11. Defrost and centrifuge one mRNA aliquot in an Eppendorf centrifuge at maximum speed (16,000 $\times g$) at 4°C for 15–45 min. Keep on ice.
12. Prepare the microinjection needle and load it with 0.5–1.5 μL of mRNA solution.

Note: We use home-made microinjection needles made from glass capillaries (outer diameter 1 mm, inner diameter 0.75 mm, length 100 mm, purchased from Harvard apparatus) with the help of a Narishige PN-31 puller (currently discontinued, possible alternative is Narishige PC-100). The puller settings have to be determined experimentally as identical settings on identical machines do not result in the same needle configuration.

Note: Load the needle with 0.5–1.5 μ L of mRNA solution with the help of microloader tips (Eppendorf).

13. Place a 10–15 μ L drop of the culture medium with dbcAMP in the center of a glass slide with a shallow depression and cover with a small amount of mineral oil. Transfer the oocytes into this drop.

Note: Make sure that the mineral oil covers the drop of the culture medium completely. This prevents the medium from evaporating which would change the osmotic pressure and affect the oocyte quality. Glass slides with a shallow depression (“depression glass slides” or “cavity glass slides”, for example Globe Scientific ref. #1341-72 or VWR ref. # 470235-728) are perfect for this purpose. If the medium drop is not covered with the mineral oil completely you can also reduce the volume of the drop.

14. Place the glass slide with oocytes under the microinjection microscope, lower the holding pipette and the loaded microinjection needle to the oocyte level.
15. Switch the microinjection pump to constant flow setting (Figures 1A and 1B).

Note: We use Eppendorf FemtoJet Microinjector with constant flow setting as it is the best way to ensure oocyte microinjection success. It does not deliver an exact, predetermined volume of mRNA into the oocyte. However, the amount of protein expression after microinjection has to be measured by other means anyway, as protein expression does not only depend on the amount of mRNA injected, but also on its quality and ternary structures it may form.

16. Capture one oocyte with the holding pipette and insert the tip of the microinjection needle in the oocyte, avoiding the nucleus (Figure 1C). Quickly take the needle out after injection.
17. Release the oocyte from the holding pipette.
18. Repeat steps 16 and 17 to microinject all the oocytes.

Note: For holding pipettes, we use VacuTip microcapillaries, inner diameter 15 μ m, outer diameter 100 μ m, angle 35°. The pipette is connected to an Eppendorf CellTram Oil manual microinjector. For an example of a microinjection setup, see Figure 1.

Note: Successful injection is indicated by a short burst in the cytoplasm, the size of the burst depends on the pressure supplied to the microinjection needle. Increase or reduce the pressure setting of the Microinjector to change the injection force. Ideally the size of the burst should be comparable or smaller than the oocyte nucleus.

Note: After injection, retrieve the needle quickly but carefully, keeping the same angle so not to tear the plasma membrane and kill the oocyte.

Note: We arrange oocytes under the microscope in a way that not injected oocytes are “above” and injected oocytes are “below” the microinjection needle. With the holding pipette, one oocyte after the other is taken from “above”, moved in front of the microinjection needle, injected, and moved to the “lower” pool of injected oocytes.

Note: Depending on the microinjection skills of the user and the amount of injected mRNA, 30%–80% oocytes will survive, others will lyse within 15–30 min after injection.

19. Transfer the injected oocytes from the glass slide into an unused drop of medium in the original culture dish.
20. Incubate at 37°C for 1–4 h to ensure that there is sufficient time for protein expression.

Note: Depending on mRNA concentration, the length of the mRNA to be translated and efficiency of protein expression, the incubation time can be shortened or extended. However, too long incubation may interfere with efficient prophase I exit after dbcAMP washout.

Release from cell cycle arrest and timelapse imaging

⌚ Timing: 10–12 h

The injected oocytes are released from prophase I arrest and prepared for timelapse imaging in presence or absence of Aurora B/C inhibitors. Oocytes are filmed to capture metaphase I to anaphase I transition.

21. Prepare a culture dish with 5–6 drops (around 30 μ L each) of culture medium without dbcAMP and cover with mineral oil. Pre-warm the dish at 37°C.
22. Place the injected oocytes into several successive drops of culture medium, thus removing trace amounts of dbcAMP to allow cell cycle resumption.
23. Incubate the oocytes at 37°C for 50–70 min until they start undergoing germinal vesicle breakdown (GVBD).
24. Ten to fifteen minutes after the first oocytes undergo GVBD, discard all oocytes still in prophase.

Optional: Prepare a dish with 3–4 drops (around 30 μ L each) of medium containing Aurora B/C (or Aurora A) inhibitor (ZM447439 or AZD1152 for Aurora B/C and MLN 8237 for Aurora A), cover with mineral oil, and prewarm the dish at 37°C. Transfer the required number of injected oocytes that underwent GVBD on time through subsequent pipetting into 2–3 drops of medium with inhibitors, leaving them to incubate in the last drop.

Note: GVBD marks oocyte exit from prophase and resumption of meiosis. The most competent and healthy oocytes undergo GVBD approximately 1 h after release from dbcAMP-containing medium, which maintains them arrested. It is possible to use the oocytes that undergo GVBD after the first batch, but these oocytes are more likely to have no or delayed anaphase I onset. We never use oocytes that undergo GVBD more than 90 min after release.

Note: Aurora B/C is required for the so-called error correction pathway, to detach wrongly attached microtubule fibers. It is functional during early meiosis, when microtubules attach and detach continuously. The error correction pathway signals to the spindle assembly checkpoint (SAC) which imposes a cell cycle delay. As a consequence, inhibition of Aurora B/C results in accelerated cell cycle progression. Under normal conditions, mouse oocytes of the strain backgrounds we use undergo anaphase I around 7–9 h after GVBD, and 4.5–5 h after GVBD if the SAC is inactivated. Thus, adjust timelapse imaging setup to account for accelerated anaphase timing in presence of Aurora B/C inhibitors.

Note: The concentration of AuroraB/C inhibitors should be determined experimentally as we found that depending on the manufacturer and production batch the quality may differ. Too strong AuroraB/C inhibition affects chromosome condensation, making them appear as a uniform mass. It is impossible to correctly measure the cleavage efficiency of the sensor in this

case. Also, too high concentration may lead to unspecific inhibition of Aurora A, preventing anaphase I onset.

The aliquots of the inhibitors should be prepared according to the manufacturer's instructions and diluted in the culture medium to obtain the desired concentration just before use.

Note: AuroraB/C is required for cytokinesis, i.e., polar body (PB) extrusion. Anaphase I onset is detectable through rapid chromosome movements, or an attempt to extrude a PB which is then retracted.

25. Incubate the synchronized batch of oocytes at 37°C for 4–6 h.
26. Prepare a microscopic chamber such as suggested by the microscope manufacturer with multiple drops of 2 μ L medium and cover with mineral oil. Prewarm at 37°C.
27. Place 1–2 injected oocytes in each drop.

Note: If using Aurora kinase inhibitors, use medium containing inhibitors when preparing the chamber.

Note: Oocytes are very sensitive to laser irradiation, such as used in confocal imaging systems. To reduce laser exposure, it is best to place individual oocytes apart from each other. The number of medium drops in the chamber should accommodate the number of oocytes in the experiment. The maximum number of oocytes depends on the conditions and is best determined experimentally.

28. At 4–6 h after GVBD, start timelapse imaging: use the transmitted light, the 491 nm and 561 nm channels, acquire a z-stack of the region around the chromosomes (30–45 μ m with a step-size of 3 μ m), with acquisitions every 15–20 min.

Note: Duration of timelapse imaging should start in metaphase I and cover anaphase I, which normally occurs 7–9 h after GVBD. It may be slightly delayed (<30 min) due to the imaging conditions, compared to the oocytes kept in the incubator. A more significant delay indicates that culture conditions are suboptimal.

Note: Start of the movie has to be adapted to accelerated cell cycle progression in Aurora B/C inhibitor-treated oocytes.

29. After timelapse imaging check that the oocytes have survived and control oocytes have extruded a polar body, indicative of exit from meiosis I.

Cleavage efficiency quantification

⌚ Timing: 2 h

The movies are analyzed and biosensor cleavage efficiency is quantified.

30. Using ImageJ, open image sequences (as stacks and hyperstack) for TRANS, 491 nm and 561 nm channels for one oocyte.
31. For measuring, select the oocytes with their chromosomes fully visible during several timepoints before, during and after anaphase I.

Note: The chromosomes may migrate out of focus during anaphase if the metaphase plate is not parallel to the plane of imaging. The signal from only partially visible chromosomes should be discarded, as it will introduce errors in quantifications.

32. Identify the timepoint of anaphase I (marked by chromosome segregation and beginning of polar body extrusion).

Note: If Aurora B/C kinases are inhibited, chromosome segregation is often problematic as endogenous Rec8 cleavage depends on the activity of these kinases. In this case, anaphase can also be spotted through the attempt of the oocyte to extrude a polar body (which will most likely retract) in the transmitted light channel, or a sudden movement of the chromosomes towards the oocyte cortex (in the 561 nm channel). Consider the timepoint where either of these events occur as anaphase, and keep in mind that oocytes devoid of functional Aurora B/C undergo anaphase I onset several hours earlier compared to untreated oocytes, due to absence of error correction signaling to the SAC.

33. Starting from 3–4 timepoints before anaphase and until 3–4 timepoints after anaphase, measure mean fluorescence on Z-projection (maximum intensity) in 491 nm and 561 nm channels in a box that includes all chromosomes ("Chromosomes") and in another box placed in the cytoplasm away from the chromosomes ("Cytoplasm"). See [Figure 2A](#).

Note: The area measured must be the same in size and position between the two channels. As the chromosomes will change position during anaphase, make sure to follow the chromosomes with the area that is being measured.

Note: During anaphase, chromosomes are separated with half of them being extruded into the PB. When the area measured cannot cover both halves anymore, shift the area to cover only one half of the now segregated chromosome masses, preferably the one remaining in the oocyte, but keep in mind that the signal has now been divided by two ([Figure 2](#)).

34. Import the measurements into MS Excel, subtract the "Cytoplasm" from the "Chromosomes" signal for each channel and for each timepoint.
35. Normalize the result for each channel separately to the timepoint right before anaphase ([Figure 2B](#)).

Note: If the value at the timepoint right before or at onset of chromosome segregation is significantly lower than the value at the timepoint just before, take the higher value for normalization. (Separase cleavage may have started already before segregation of chromosomes is visible).

36. Calculate the ratio between the values obtained for the 491 nm and 561 nm channels (divide the normalized value of the 491 nm channel by the normalized value of the 561 nm channel for each timepoint).
37. Find the lowest post-anaphase value (within 0–30 min after anaphase), and subtract it from the highest pre-anaphase value. This value represents the portion of the sensor that was cleaved during anaphase, i.e., cleavage efficiency of the sensor.

Note: Cleavage efficiency is notoriously variable between oocytes, especially if biosensor expression levels are low. Hence, it is important to quantify sensor cleavage in a high number of oocytes to obtain statistically significant results.

EXPECTED OUTCOMES

Cleavage efficiency of a sensor is a numerical value representing how many molecules of the biosensor were cleaved in a separase-dependent manner at anaphase I onset. The values should present a statistically significant difference when compared to the values measured from the

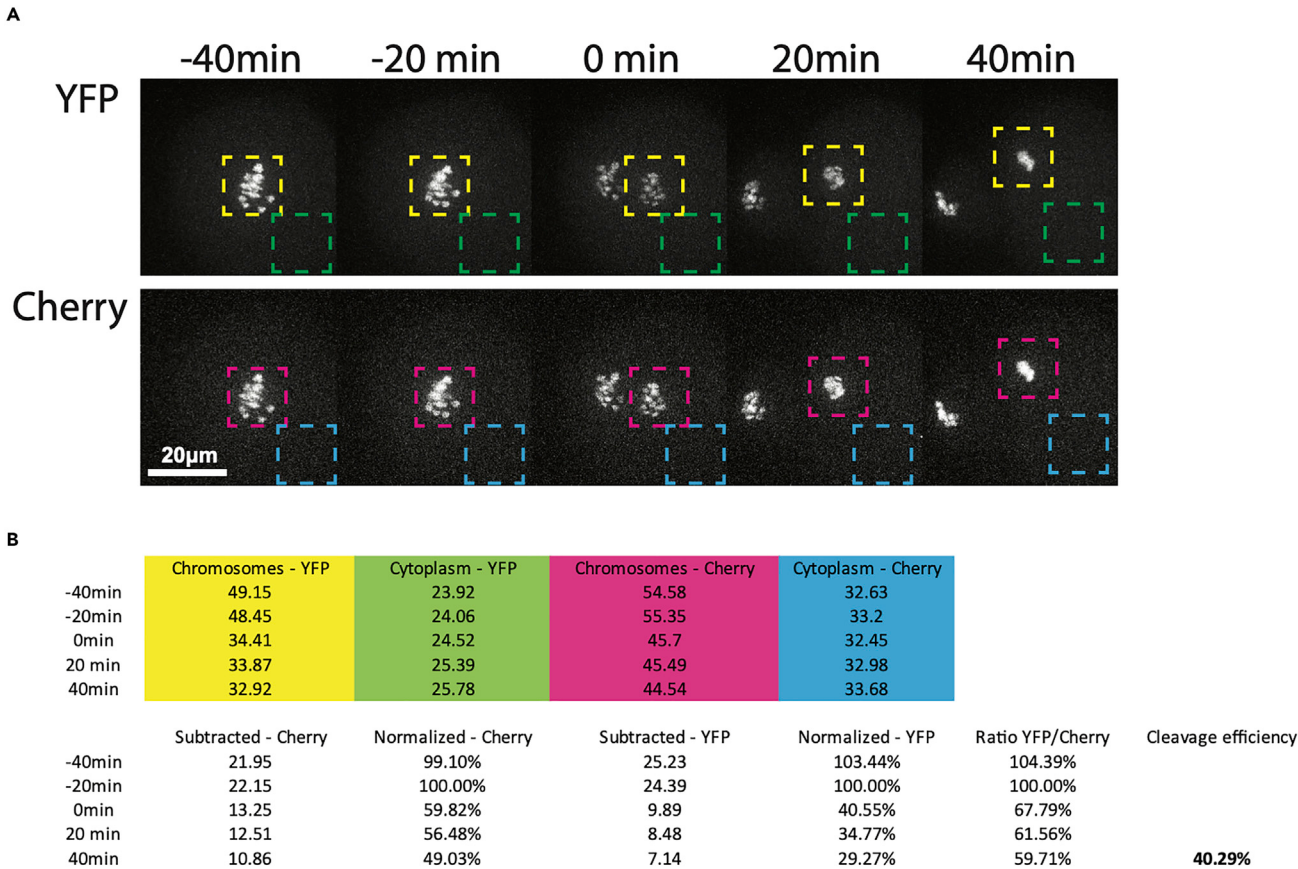


Figure 2. Measuring biosensor cleavage efficiency from timelapse images

(A) A montage of timelapse images of a mouse oocyte undergoing anaphase I (marked as 0 min). Rectangles of colored dashed lines mark the areas where the mean fluorescence signal should be measured. Scale bar: 20 µm.

(B) Calculations based on the measurements from (A), colored blocks correspond to measurements from the rectangles of colored dashed lines.

negative control (a sensor without a separase cleavage consensus sequence). It is expected that the cleavage efficiency is different between oocytes based on the level of biosensor expression and noise-to-signal ratio in a given oocyte.

It is expected that inhibition of Aurora B/C but not Aurora A activity reduces or completely inhibits cleavage of the Rec8-containing biosensor. In absence of separase or separase activation, no cleavage should be observed.

LIMITATIONS

The method for measuring Rec8 cleavage efficiency we describe here is best suited for mouse oocytes and not in other experimental systems, such as cultured somatic cells. The use of kinase inhibitors such as described here for Aurora kinases can be challenging due to possible side effects perturbing chromosome structure, chromosome segregation, or cell cycle progression. It is essential to confirm the conditions for this protocol with positive and negative controls, like Scc1-containing biosensor (positive control) or non-cleavable biosensor (negative control).

TROUBLESHOOTING

Problem 1

Oocytes do not go through anaphase during timelapse imaging (step 29).

Potential solution

- Oocytes are very sensitive to the temperature, quality of culture medium and the quality of the mineral oil that is used to cover the medium droplets. Test whether the oocytes complete the first meiotic division normally in the incubator at 37°C. If the oocytes survive and extrude a polar body at the correct time in the incubator but not under the microscope, the temperature should be checked first, preferably right before and right after the imaging session. To check the temperature of the imaging setup, we use a thermometer with a long wire sensor placed exactly where the oocytes are supposed to be (middle of the Fluorodish, right above the microscope objective).

In our experience, the temperature is less stable in the heated chamber of the microscope than in the tissue culture incubator, likely due to insulation problems or temperature changes in the area surrounding the microscope. Often the actual temperature of the culture medium droplet in the imaging chamber is different from the temperature settings of the microscope heating system. Adjust the temperature settings and make sure the actual temperature in the medium droplet is 37°C.

- During timelapse imaging, too much exposure to laser radiation can damage oocytes resulting in the failure to complete cell division. To check whether the laser irradiation is excessive, make a test run of the experiment without 491 nm and 561 nm channels, imaging with transmitted light only. If the oocytes complete anaphase I with exposure to transmitted light but not when the lasers are used, limit exposure to laser damage by reducing either laser power, exposition time, number of timepoints or number of stage positions. These settings can vary a lot from microscope to microscope. Imaging not more than 10 oocytes with timepoints every 20 min, 15 z-slices with 3 µm distance between z-slices should be a good starting point. Laser power and exposure times should be as low as possible, but sufficient enough to clearly visualize all chromosomes. Start imaging not earlier than 1 h before anaphase I is expected, to avoid unnecessary exposure before the crucial time point you are interested in.
- Check that oocyte collection and handling techniques applied are not damaging the oocytes. Oocytes are also sensitive to the quality of mineral oil used to cover medium droplets. The oil can be additionally purified by vortexing an aliquot of oil together with milliQ water and then letting the phases separate. Other factors may include quality of the culture medium or the material of the imaging chamber.
- Certain inhibitors will block cell cycle progression. Depending on the mechanism, it is possible to override the arrest. For example, SAC-dependent arrest can be lifted with the Mps1 inhibitor Reversine.

Problem 2

Oocytes do not survive microinjection (step 18).

Potential solution

- Proper microinjection technique is required to achieve good survival rate. Practice microinjection with water and limit the injected volume. Adjust the length and shape of the microinjection needle tip by changing the settings of the needle puller. See [Figure 1C](#) for an example of the needle shape.
- Dirt on the microinjection needle tip might damage the oocyte membrane during injection. The needle tip can be cleaned by tapping or sliding it against the holding pipette tip.
- If the RNA solution is too viscous and causes needle clogging, you can dilute the solution with nuclease-free water (however, this may also reduce biosensor expression) and microcentrifuge the sample again. Make sure the RNA sample is purified.

Problem 3

Insufficient sensor expression, chromosomes are not visible (step 28).

Potential solution

- Check that the oocytes are injected by comparing background fluorescence in 491 nm or 561 nm channels of injected and not injected oocytes.
- Synthesize a fresh aliquot of mRNA, make sure not to introduce nucleases (from dust, skin) until injection.
- Increase RNA concentration, increase RNA quality by adding extra capping solution, GTP or extra polyadenylation, according to the [manufacturer's instructions](#).
- Prolong incubation time after injection.
- Verify mRNA quality of the aliquot that has been used for injection, to be sure the mRNA remained stable throughout the microinjection procedure.

Problem 4

Aurora B/C inhibition produces unexpected results (after step 24, optional).

Potential solution

- Make sure to add the inhibitors within the first 15 min after GVBD.
- Try inhibitors from different suppliers, always use single-use aliquots.
- Verify that the inhibitor works (by staining for the Aurora B/C substrate pH3S10, and checking that anaphase I onset is accelerated).
- If the chromosomes are decondensed and stuck together before anaphase I, try reducing the concentration of the inhibitor or changing suppliers.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed and will be fulfilled by the lead contact, Katja Wassmann (katja.wassmann@ijm.fr).

Materials availability

All unique reagents generated in this study are available from the [lead contact](#) without restriction. The study did not generate new mouse lines.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

E.N. and K.W. designed the concept, E.N. did experimental work and troubleshooting, E.N. wrote the manuscript with input from K.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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