Sep 02, 2021

Microinjection Techniques in Fly Embryos to Study the Function and Dynamics of SMC Complexes

Book Chapter

Catarina Carmo, Margarida Araújo, Raquel Oliveira

dx.doi.org/10.17504/protocols.io.bnx6mfre

Springer Nature Books

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ABSTRACT

Structural maintenance of chromosomes (SMC) proteins are critical to maintain mitotic fidelity in all organisms. Over the last decades, acute inactivation of these complexes, together with the analysis of their dynamic binding to mitotic chromatin, has provided important insights on the molecular mechanism of these complexes as well as into the consequences of their failure at different stages of mitosis.

Here, we describe a methodology to study both SMC function and dynamics using *Drosophila melanogaster* syncytial embryos. This system presents several advantages over canonical inactivation or imaging approaches. Efficient and fast inactivation of SMC complexes can be achieved by the use of tobacco etch virus (TEV) protease in vivo to cleave engineered versions of the SMC complexes. In contrast to genetically encoded TEV protease expression, *Drosophila* embryos enable prompt delivery of the protease by microinjection techniques, as detailed here, thereby allowing inactivation of the complexes within few minutes. Such an acute inactivation approach, when coupled with real-time imaging, allows for the analysis of the immediate consequences upon protein inactivation. As described here, this system also presents unique advantages to follow the kinetics of the loading of SMC complexes onto mitotic chromatin. We describe the use of *Drosophila* embryos to study localization and turnover of these molecules through live imaging and fluorescence recovery after photobleaching (FRAP) approaches.

DOI

dx.doi.org/10.17504/protocols.io.bnx6mfre

EXTERNAL LINK

https://link.springer.com/protocol/10.1007/978-1-4939-9520-2_19

PROTOCOL CITATION

Catarina Carmo, Margarida Araújo, Raquel Oliveira 2021. Microinjection Techniques in Fly Embryos to Study the Function and Dynamics of SMC Complexes. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bnx6mfre

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Carmo C., Araújo M., Oliveira R.A. (2019) Microinjection Techniques in Fly Embryos to Study the Function and Dynamics of SMC Complexes. In: Badrinarayanan A. (eds) SMC Complexes. Methods in Molecular Biology, vol 2004. Humana, New York, NY. https://doi.org/10.1007/978-1-4939-9520-2_19

KEYWORDS

SMC complexes, Cohesin, Condensin, Drosophila melanogaster, Syncytial embryo, Microinjections, TEV protease, Fluorescence recovery after photobleaching (FRAP), FRAP

Citation: Catarina Carmo, Margarida AraÃÂºjo, Raquel Oliveira (09/02/2021). Microinjection Techniques in Fly Embryos to Study the Function and Dynamics of SMC Complexes. https://dx.doi.org/10.17504/protocols.io.bnx6mfre

CREATED

Oct 25, 2020

LAST MODIFIED

Sep 02, 2021

OWNERSHIP HISTORY

Oct 25, 2020 Lenny Teytelman protocols.io

Jul 05, 2021 Emma Ganley protocols.io

Aug 24, 2021 Satyavati Kharde

Aug 26, 2021 satyavati Kharde

PROTOCOL INTEGER ID

43742

GUIDELINES

Introduction

For life to be sustained over time, its basic unit—the cell—needs to ensure its successful division into two daughter cells by correctly duplicating its genome and equally segregating it. Structural maintenance of chromosomes (SMC) complexes, particularly cohesins and condensins, are critical players for the fidelity of this process. Cohesin holds sister chromatids together until the metaphase to anaphase transition, favoring proper chromosome attachment to mitotic spindle [1]. In turn, condensin complexes are important to maintain both mitotic chromosome structure and sister chromatid resolution [2]. This is thought to be dictated by topological entrapment of DNA molecules by SMCs (for review *see* [3, 4, 5]). Although much is known about the role of these complexes during mitosis, the exact mechanism for their action and chromatin association, as well as how the entire mitotic apparatus responds to their inactivation, is not yet fully understood.

The use of *Drosophila melanogaster* syncytial embryos for the study of mitosis has long been a valuable and useful system in which it is possible to follow the dynamics and function of essential proteins at a particular phase of the mitotic cycle [6]. Notably, the first nuclear divisions in *D. melanogaster* syncytial embryos are synchronous and quite fast (average 8 min) until the mitotic cycle 14. In this window of embryonic development, the chromatin state is the most naïve as it can be, as it is devoid of transcription (maternal RNAs/proteins are deposited in the egg) and other confounding effects associated to gene expression [7]. Additionally, *D. melanogaster* is a model system for which there is a wide range of genetic tools already available, including a fairly high number of different mutant and transgenic lines which express functional fluorescently tagged mitotic proteins.

In addition to the classical usage of this powerful system to study protein function, recent developments enable the use of these embryos to study the immediate consequences of protein inactivation, using a TEV-mediated protein cleavage. Canonical studies rely on the inactivation of mitotic proteins prior to entry in nuclear division (e.g., using genetic KO or RNAis). These approaches have the caveat of being slow and often incomplete. For more acute and efficient inactivation, genetically engineered TEV-cleavable SMC complexes can be generated by introducing TEV recognition sites into specific regions of these molecules, mostly within the linker of the kleisin subunit (Fig. 1). Upon TEV protease-mediated cleavage, the integrity of the SMC complex is lost, thereby triggering its inactivation. TEV protease-mediated cleavage of SMC complexes was originally performed for cohesin in yeast cells [10] and later adapted to *D. melanogaster* [8], mouse oocytes [11], and human cells [12]. Subsequent studies used similar approaches to inactivate condensin complexes in yeast [13], mouse oocytes [14], and *Drosophila* [9].

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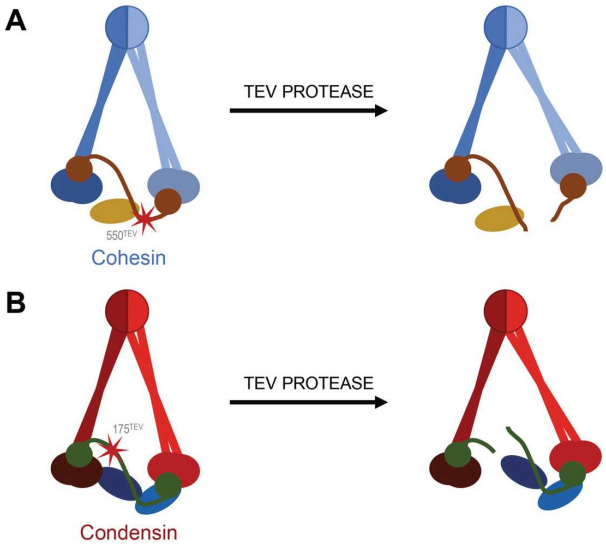

Fig. 1
TEV-cleavage system for cohesin and condensin complexes in *Drosophila melanogaster*. (**a**) Schematic of the cohesin complex containing TEV-cleavable Rad21/Scc1 (brown), SMC3 (dark blue), SMC1 (light blue), and Scc3/SA (yellow). Star shows the site of insertion of TEV recognition sequences (numbers refer to amino-acid positions), adapted from [8]. (**b**) Schematic of the condensin I complex with the TEV-cleavable Cap-H/Barren (green), SMC2 (dark red), SMC4 (light red), and CAP-D2 and CAP-G (blue tones). Star shows the site of insertion of TEV recognition sequences (numbers refer to amino-acid positions), adapted from [9]

The efficiency and acuteness of TEV-mediated protein inactivation, coupled with microinjection techniques to enable prompt delivery of TEV, allow for the analysis of the role of specific proteins in the maintenance of particular states (e.g., chromosome organization) rather than in their establishment. In fly syncytial embryos, TEV protease injection enables full inactivation of Cohesin within 2 min and of Condensin I in ~15 min [9, 15]. Using this approach, previous studies revealed that cleavage of cohesin is sufficient to induce individualization of sister chromatids although efficient anaphase movements require further changes in the cell cycle state [15]. This approach has been recently modified to enable removal of well-defined amounts of cohesin complexes from metaphase chromosomes [16]. This quantitative analysis revealed that sister chromatid cohesion is very resistant to cohesin loss yet partial cohesin decay compromises chromosome attachments. More recently, TEV-mediated inactivation has also been used to inactivate condensin I complexes from previously established chromosomes, specifically in metaphase [9]. Acute metaphase cleavage of condensin I, the major condensin complex in *Drosophila*, results in disassembly of the centromere-proximal regions. Most chromatin mass, in turn, undergoes de novo chromatin intertwining caused by topoisomerase II-dependent re-entanglements. This leads to overcompaction of chromosomal arms and ultimately failure of chromosome segregation [9]. Although most studies using TEV protease inactivation have been focused on SMC complexes, this technology holds the prospect of being more widely applicable and recent studies were successful at adapting this system to inactivate *Drosophila* kinesin 5 [17]. In addition to protein inactivation, study of the dynamic association of SMC complexes with chromatin has also highlighted important aspects for their mode of action. Fluorescence recovery after photobleaching (FRAP) was introduced decades ago and today is probably the most widely used method to study protein dynamics in a multitude of contexts. Its foundation is based on the irreversible photobleaching (transition of a fluorophore into a nonfluorescent molecule) of fluorescent molecules by intense light excitation. The natural diffusion of these molecules makes it possible for the exchange of bleached molecules within the FRAPed region of interest (ROI), until an equilibrium is reached [18]. Monitoring the kinetics of fluorescence recovery of a given protein with a GFP tag (or similar, e.g., YFP and mCherry), over time, allows for the assessment of how mobile this protein is. Immobile proteins will not exchange with the unbleached molecules and thereby no recovery should be detected. In contrast, for proteins with high turnover, unbleached molecules will quickly replace the bleached fraction, leading to recovery of the fluorescence within the bleached region.

FRAP studies identified multiple pools of cohesin whose stability on chromatin varies during the cell cycle. Cohesin complexes involved in sister chromatid cohesion are known to be stably associated with chromatin, whereas the pool involved in other noncanonical functions of cohesin (e.g., regulation of gene expression) turns over within seconds to minutes [19, 20]. On the other hand, condensin complexes display different properties during mitosis: condensin II is mostly stably associated with mitotic chromatin, whereas condensin I turns over within a few minutes [21, 22]. The dynamic nature of condensin I inactivation with mitotic chromatin contrasts with classical models where these complexes are statically holding chromatin loops and inspired new models for how these

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complexes may shape chromatin in a more dynamic manner [23, 24]. *Drosophila* embryos present unique advantages to study the dynamics of chromatin binding proteins. In particular, these divisions are very rapid, offering a high mitotic index per sample analyzed. More importantly, these divisions are highly synchronous and can be arrested at multiple stages, thereby enabling the concomitant analysis of multiple nuclei. Lastly, all nuclei share a large common cytoplasm that renders the bleached molecules negligible when performing FRAP studies.

In this chapter, we describe how to perform microinjection of specific proteins into *Drosophila* embryos, using TEV-mediated cleavage of SMC complexes as a proof of principle. The methodology, however, can be used to deliver any compound to these fast dividing nuclei, including small molecule inhibitors, antibodies [25], dominant negative proteins [15, 26], mRNAs [15], and TALE-lights [27, 28]. We further exemplify how microinjection approaches can aid in FRAP analysis of SMC complexes, in a time-resolved manner.

Acknowledgments

We would like to thank the technical support from Instituto Gulbenkian de Ciência's Advanced Imaging Facility, supported by national Portuguese funding (PPBI-POCI-01-0145-FEDER-022122), and the Fly Facility, supported by the Congento Consortium (LISBOA-01-0145-FEDER-022170). These two programs are supported by Lisboa Regional Operational Program (Lisboa 2020) under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER) and Fundação para a Ciência e a Tecnologia (FCT; Portugal).

C. Carmo and M. Araújo are supported by FCT PhD scholarships (PD/BD/128428/2017 and PD/BD/128431/2017, respectively). The R.A. Oliveira lab is supported by an FCT Investigator grant (IF/00851/2012/CP0185/CT0004), a European Molecular Biology Organization Installation Grant (IG2778), and a European Research Council Starting Grant (ERC-2014-STG-638917).

Supplementary material

Movie S1: https://link.springer.com/protocol/10.1007/978-1-4939-9520-2_19#SupplementaryMaterial
Microinjection in a Drosophila embryo. Example of a microinjection procedure. Embryo was injected with a green fluorescence dye for illustrative purposes. Video shows DIC image merged with fluorescence imaging on the green channel (to visualize the dye). Note membrane retraction upon needle contact, which should be followed by a continuous movement into the embryo. Once injection takes place, the injected solution diffuses into the cytoplasm (mp4 359 kb)

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MATERIALS TEXT

Instruments, Disposables, and Equipment:

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1. Fly cages, small.
2. Artist's brush.
3. Scalpel.
4. Stainless steel probes, tip diameter 0.25 mm (FST 10140-01).
5. Cell strainer 100 µm, Nylon.
6. Microscope coverslips (18 × 18 mm or 22 × 22 mm and 24 × 60 mm) and compatible microscope inserts.
7. Small containers (similar to a tip box).
8. Squeeze bottle with H_2O.
9. Prepulled Femtotips microinjection capillary needles 0.5 × 1.0 µm (Eppendorf).
10. Femtotips Microloader Tips (Eppendorf).
11. Regular stereo microscope, appropriate for dissection.
12. Microinjector Controller (Eppendorf FemtoJet microinjector or similar).
13. Micromanipulator with three-axis piezo movement, equipped with a pipette holder mount and adapted to a fluorescence microscope.
14. Fluorescence microscope (spinning disk, confocal, or wide-field) with 10× or 20× (for injection) and 63× or 100× objectives (for live cell imaging).

Reagents and Stock Solutions:

1. Apple juice agar plates.
2. Baker's yeast—Prepare a thick paste by diluting yeast with water. Store it at 4 °C and keep it for under a week.

Take care with the thickness of the yeast paste: it should not be too liquid, otherwise flies will stick to it.

3. 50% (v/v) bleach—Prepare commercial hypochlorite solution in water, fresh for each day.
4. Halocarbon oil 700.
5. Heptane and double-faced tape, to produce glue: In a 250 ml glass bottle, put double-faced tape enough to fill the container. Add enough heptane so as to completely fill the container. Close the bottle and leave it overnight. Remove a few ml of the liquid and filter it into a small glass container. Use small volumes for a working batch, as the heptane will evaporate with each opening of the vial and make the glue thicker.

When the working heptane glue solution becomes thicker, add a few drops of heptane or make a new working batch from the stock bottle. Heptane glue should be transparent so that it does not interfere with imaging.

6. Proteins and other reagents to inject:
(a) TEV-mediated inactivation: 5–10 mg/ml TEV protease, purified from *E. coli*, in 20 mM Tris–HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, and 2 mM DTT.

Keep protein and drug stocks in small-volume aliquots (~4 µl) at −80 °C for long-term storage. The working aliquot can be stored at −20 °C and must be kept on ice during experiments.

For purification details *see* refs. [15, 32].

(b) Metaphase arrest: 12–30 mg/ml UbcH10^{C114S}, purified from *E. coli*, in 20 mM Tris–HCl pH 7.5, 300 mM NaCl; or 2 mM colchicine in 1 × PBS.

Keep protein and drug stocks in small-volume aliquots (~4 µl) at −80 °C for long-term storage. The working aliquot can be stored at −20 °C and must be kept on ice during experiments.

For purification details *see* refs. [15, 32].

Colchicine is a potent microtubule poison that fully disrupts the mitotic spindle. If the integrity of the spindle is to be preserved, UbcH10^{C114S} should be used instead. This is a dominant-negative form of the E2 ubiquitin

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ligase needed for APC/C reactions [33].

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Collecting and Preparing Embryos for Live Imaging

1 Set up a cage with the fly strain of your choosing. Use an apple juice plate with a smear of fresh yeast paste at the bottom of the cage.

When setting a cage, take into consideration that older flies have a decreased egg-laying capacity. Males that have matured for over 3 days mate more efficiently and females reach their peak of egg laying 4–7 days after eclosion.

Take into consideration that flies tend to lay more eggs during the night-time. If more convenient, cages can be kept inside a box with an inverted light–dark cycle (e.g., to become dark at 12 pm and light at 12 am). In this manner, flies will lay more eggs during the afternoon.

Set up a cage for at least a full day before the day of experiments, in order for flies to acclimatize to the cage and fully recover from CO_2 anesthesia.

As flies are attracted by the smell of fruit and eat yeast, they will be attracted to the apple juice plate with yeast paste, and lay a lot of eggs that can be collected.

2 Change plates at least once a day, even on days without experiments. To do this, invert the cage, tap it strongly, so as to bring the flies to the bottom, and exchange plates.

3 On the day of the experiments, start with a precollection of **01:00:00** – **02:00:00** to release retained eggs and increase staging accuracy. 3h

Flies tend to hold embryos before depositing them but fresh yeast paste stimulates the egg laying. Changing the plates every morning will ensure that you remove the embryos held overnight in their oviducts. This will also give a good idea of the egg-laying efficiency.

4 The time of collection will depend on the desired developmental time—shorter collection times for early divisions and longer collection times for late developmental stages. For example, to collect embryos that are, at most, at nuclear division 10 (blastoderm nuclei), corresponding to **01:30:00** of development, you may want to start with a collection of **01:15:00** , counting with ~ **00:15:00** for embryo preparation. 3h

5 To collect embryos from the agar plate (Fig. 3a), use an artist's brush (moist with water) and swipe them onto a cell strainer, placed on a container with tap water.

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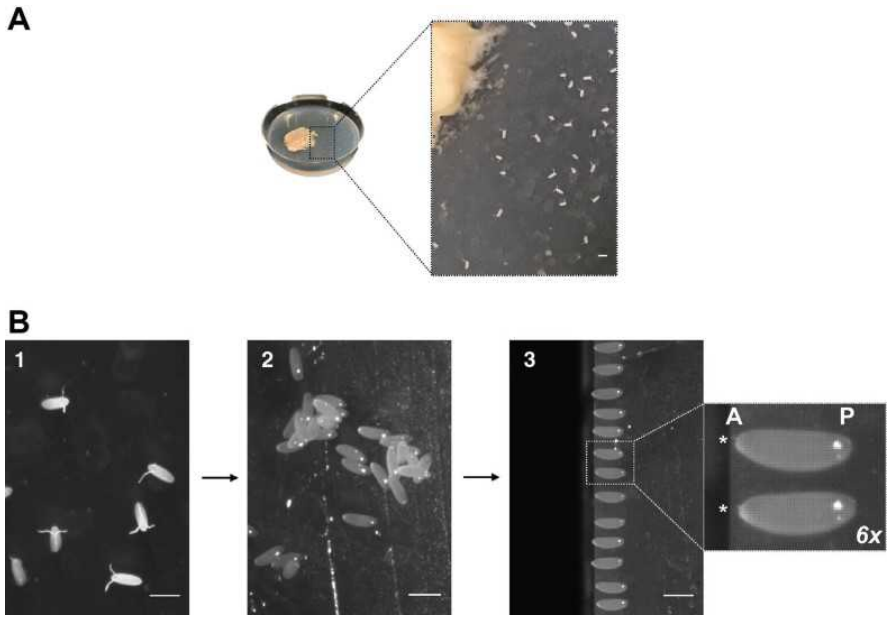

Fig. 3 Preparation of embryo samples for microinjection/live imaging.
(**a**) Embryo collection in apple juice agar plates with yeast paste (see **step 1**).
(**b**) (1) Higher magnification of A.
(**b**) (2) Embryos after dechorionation (see **step 5**).
(**b**) (3) Alignment of embryos in an agar block, before being transferred onto a previously prepared slide with heptane glue (see **steps 7–12**.)*A* and *P* indicate the anterior and posterior pole, respectively; asterisks denote the micropyle.
Scale bar: 500 µm

6 2m

Briefly remove excess water on a tissue paper and transfer the embryo containing cell strainer to a container with 50% (v/v) bleach. Incubate for **00:02:00** at **Room temperature** . This will remove nontransparent chorion (dechorionation), essential for injection and imaging.

Time left on bleach solution will depend on embryo's resistance; more fragile embryos may require less time. Also, by the end of the day, the bleach solution will become weaker and it may be required to leave embryos longer.

7 Remove excess bleach solution with a tissue paper. Wash embryos with a squeeze bottle with distilled water. Water pressure from the bottle directly on the embryos will help in the removal of chorion. Rinse the embryo containing cell strainer in the container with tap water.

Replace the water from the tap water container between collections and wash the cell strainer with the help of an artist's brush in order to get rid of remainders of bleach and embryos (from the cell strainer).

8 Cut a small block of a clean apple juice agar with a scalpel and place it on a coverslip, to be viewed under a stereo microscope.

9 Using a 24 × 60 mm coverslip, place around **6 µl** – **8 µl heptane glue** in the middle of the coverslip as a single row and tilt it to make it spread as an even layer. This will be used to mount the embryos for live imaging.
For injections: take a smaller coverslip (18 × 18 or 22 × 22 mm) and place it so as to overlay approximately half of the

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glue layer (Fig. 2 for scheme).

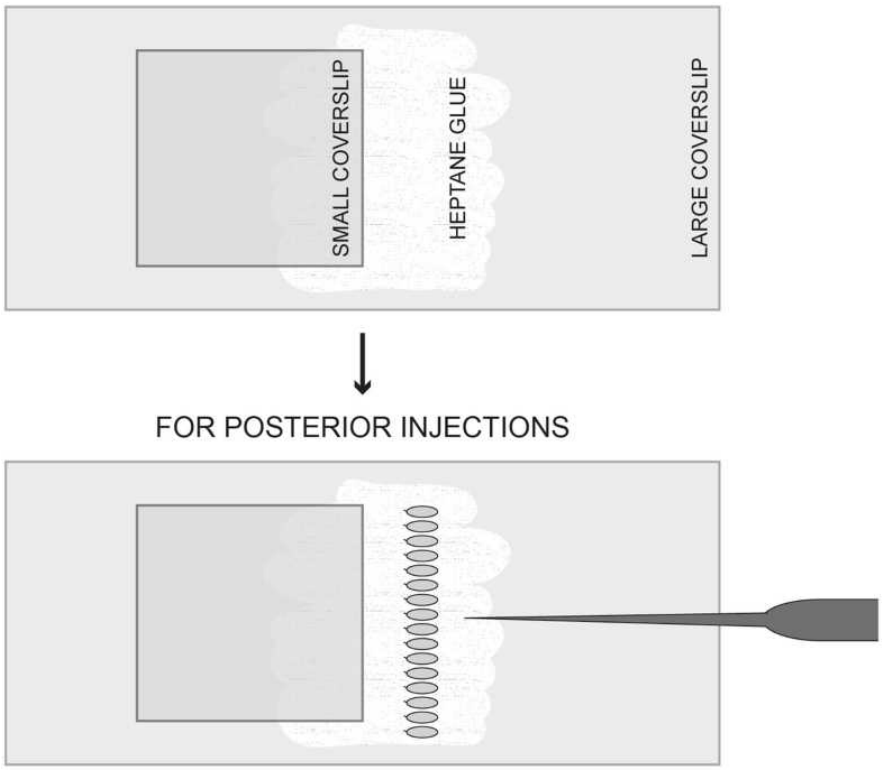

Fig. 2 Slide preparation for microinjection/live imaging.
A thin layer of heptane glue is placed in the middle of a large coverslip (e.g., 24 × 60 mm). A smaller coverslip (e.g., 18 × 18 or 22 × 22 mm) is placed on top leaving half of the glue area for the embryos. For posterior injections the coverslip should be on the left side facing the anterior pole of the embryos

10 Transfer embryos from the cell strainer using a brush and place them on the agar block, with the aid of a steel probe. Dechorionated embryos should have an ovoid shape without the dorsal appendages (Fig. 3b2).

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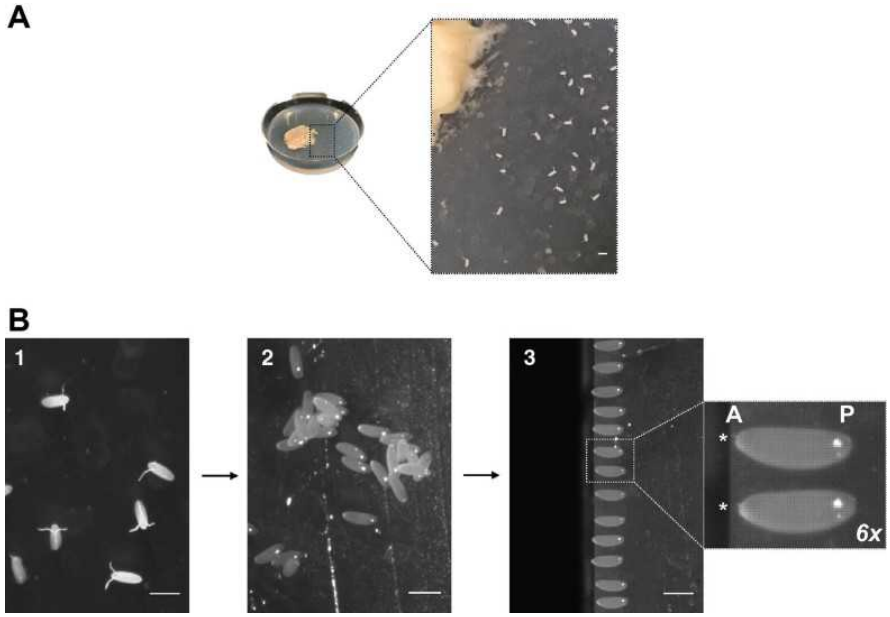
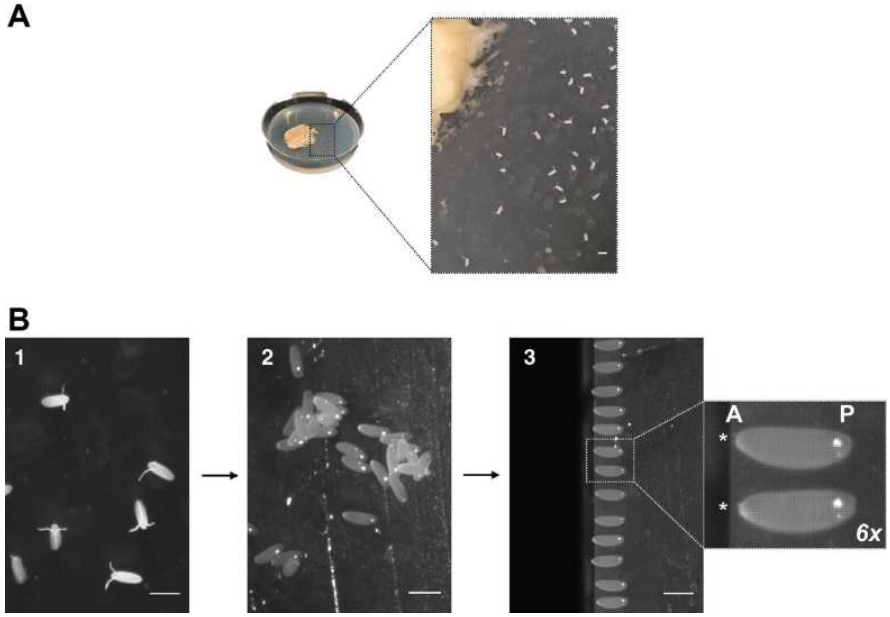


Fig. 3 Preparation of embryo samples for microinjection/live imaging.
(**a**) Embryo collection in apple juice agar plates with yeast paste (see **step 1**).
(**b**) (1) Higher magnification of A.
(**b**) (2) Embryos after dechorionation (see **step 5**).
(**b**) (3) Alignment of embryos in an agar block, before being transferred onto a previously prepared slide with heptane glue (see **steps 7–12**.)*A* and *P* indicate the anterior and posterior pole, respectively; asterisks denote the micropyle.
Scale bar: 500 µm

11 Align the embryos in a row, making sure all face the same direction (i.e., every embryo has the anterior side, marked by micropyle, oriented to the same direction). Use the edge of the agar block as a reference (Fig. 3b2).



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12 Once aligned, take the preprepared 24 × 60 mm coverslip (see step 9) and with the glue side facing down lower it until you glue the embryos, by gently pressing on the agar block. Keep it parallel to the smaller coverslip, with the micropyle facing this side (for posterior end injections).

Proper attachment of the embryos onto the coverslip is a critical step for successful injections to avoid that the embryo "escapes" the glue once the needle approaches. Embryos must, therefore, be sufficiently dry, as water can compromise their attachment.

24m

13 For injections only: leave the preparation to dry for **00:10:00** – **00:14:00** .

An extended drying step is critical for injections to decrease the osmotic pressure of the embryo and thereby prevent bursting and cytoplasmic ejection once pierced.

14 Using a 20–200 µl tip, take halocarbon oil and place it on top the row of aligned embryos and part of the smaller coverslip. This will keep embryos moist and oxygenated.

15 Samples are ready for the following processes, including live cell imaging of unperturbed embryos (see next section).

Microinjection Techniques in Fly Embryos 30m

16

For microinjection experiments, 1–1.5 h old embryos (or 0–30 min for mRNA injections) must be collected and processed according to protocol described above. Embryos should preferentially be injected (up to three consecutive injections) at the posterior side—owing to a more uniform surface while maintaining the shape and preventing extensive loss of cytoplasm. Here we describe the use of prepulled needles to ensure repeatability.

30m

17

Before usage, centrifuge your microinjection sample at high speed for **00:30:00** at **4 °C** , so as to impede precipitates to be extracted and possibly clog the injection needle.

18 Load the needles using Microloader Tips. Take care not to leave air bubbles during loading, as it will make injection impossible. Prepare all needles needed before starting the experiment, to minimize time between injections.

19 Once at the microscope, first turn on the injector controller and the micromanipulator. Then, place the first needle in the holder—must be tight to maintain correct pressure—and connect the capillary to the pressure pump afterward.

20

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Using the lower magnification objective (10× or 20×), put the needle down slowly in the focal plane of the smaller coverslip. When the needle is close to the coverslip you will start seeing a shadow through the lens (Fig. 4a).

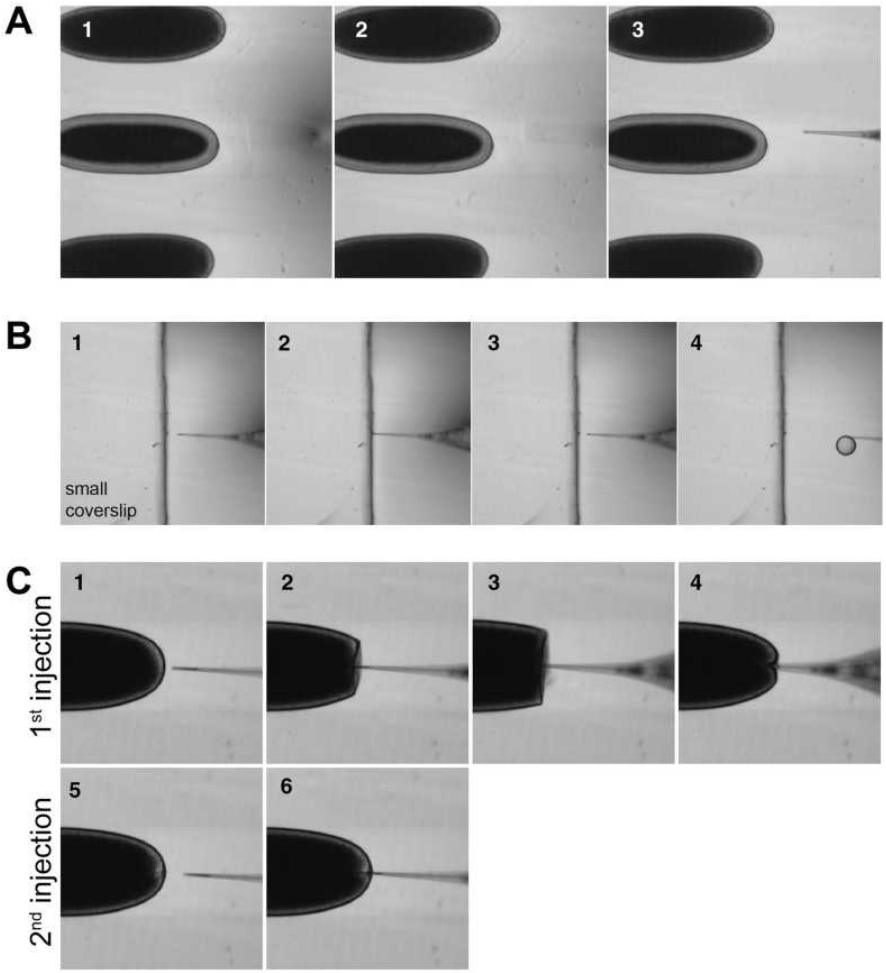

Fig. 4 Details about microinjections in *Drosophila* embryos.
(**a**) (1 –3) Bring the needle into the focal plane.
(**b**) (1–4) Open the needle with the help of the smaller coverslip and test drop size (4 shows a good-sized drop)
(**c**) Inject the embryo at the posterior pole: upon initial contact, the embryo membranes retract (2 and 3). Move the needle further until it goes through the embryo (4). 6 displays a second injection using the same injection site

21 Prepulled needles need to be further opened prior to injections. The smaller coverslip next to the row of aligned embryos will serve as a barrier to break the tip of the needle and thus to open it. Press gently the needle against the edge of the coverslip until it breaks slightly and try several injections until the correct droplet size is achieved. Press the injection button and evaluate the size of the drop (Fig. 4b), where 6 shows an appropriate drop, that should range from 30 to 50 µm in diameter (up to one-tenth of embryo length). The size of the droplet can be controlled by regulating the amount of pressure and the injection time, for example, when using an Eppendorf FemtoJet Microinjector controller. If the needle gets clogged, it can be opened further using the same strategy as before.

22 To perform an injection, as the needle comes in contact with the embryo's posterior pole, notice how the membranes retract with it (Fig. 4c2, 3 and ESM Movie S1). Move the needle further until it goes through the embryo (Fig. 4c4) and inject.

23 For multiple injections, change the needle and inject the second/third solution through the same hole. The small opening from the first injection facilitates the entry of the second needle inside the embryo, without membrane retraction. Figure 4c6 displays a second injection using the same injection site.

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Sequential injections should be performed exactly at the same site to avoid cytoplasm release. To facilitate this process, ensure a wide opening during the first injection. This can be achieved either by breaking slightly more the first needle to be used or by introducing it further inside the embryo (the wider part of the needle helps to introduce an opening which is then easier to find in subsequent injections).

If it is not possible to spot the opening, the needle can be used to probe where the injection site is by scrolling up and down slowly through the posterior side of the embryo until it gets in by itself.

Inactivation of SMC Complexes by TEV Cleavage

24

TEV-mediated inactivation requires prior establishment of *Drosophila* strains surviving solely on the TEV-cleavable version of the protein. Strains should also contain the desired fluorescent markers (e.g., H2Av- or H2B-fluorescently tagged proteins to monitor chromatin behavior). This strategy allows full and acute inactivation of targeted proteins in a time-resolved manner and thus can be applied to investigate both the establishment and maintenance of the intricate mitotic chromosome morphology. As an example of a time-restricted inactivation protocol, we detail the steps for cohesin inactivation in metaphase-arrested embryos, as originally described in [15].

The experimental layout described here focuses on the use of TEV protease to study the role of SMC complexes in the maintenance of metaphase chromosome structure. Canonical studies on the role of these complexes for the establishment of chromosome architecture can be achieved by injection of TEV during interphase, leading to precocious sister chromatid disjunction (for cohesin) or impaired sister chromatid resolution (condensin) [9, 15], depending on the question to be addressed.

25

Use your reference channel (e.g., fluorescent histones) to select an embryo with the required nuclear density and in late interphase using a 63× or 100× lens.

26

Switch to a lower magnification lens for injections.

27

14m

Induce a metaphase arrest through the injections of [M]**12 mg/ml** – [M]**30 mg/ml UbcH10C114S** into the embryo.

Imaging acquisition can be performed (using a 63× or 100× lens). After **00:06:00** – **00:08:00** , every nucleus should have their chromosomes aligned forming the metaphase plate (Fig. 5a).

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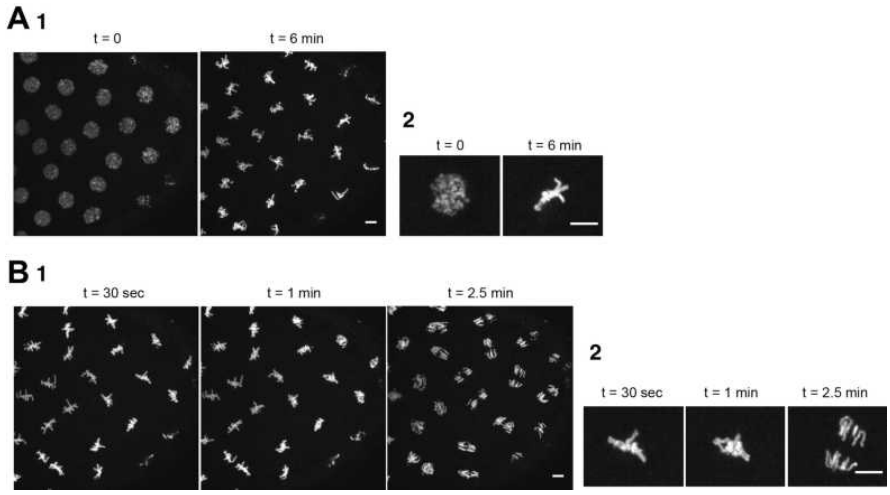

Fig. 5 TEV-mediated inactivation of cohesin during metaphase.
(**a**) and (**b**) are still images from time lapse movies in which two sequential injections were performed.
(**a**) Injection of 18 mg/ml of UbcH10^{C114S} to induce a metaphase arrest. (1) Embryos are injected during interphase (t = 0) and arrest in the subsequent metaphase (t = 6 min). (2) Crop from previous stills showing a single nucleus at t = 0 and t = 6 min after injection with UbcH10^{C114S} respectively.
(**b**) (1) Nuclei arrested in metaphase, after injection with TEV protease (12 mg/ml); sister chromatid separation is observed within 1–2 min.
(**b**) (2) Crop from previous stills showing a single nucleus at t = 30 s, t = 1 min and t = 2.5 min after TEV protease injection, respectively. Live imaging was performed using a confocal spinning disk microscope with MetaMorph acquisition software, using a 100× immersion (oil) objective. Time-lapse series were processed using Fiji.
Scale bar: 5 µm

28 3m

Subsequently, perform a second injection with TEV protease at [M]**5 mg/ml** – [M]**10 mg/ml** . If the protease is at this concentration, sister chromatid separation should be observed within **00:01:00** – **00:02:00** after TEV protease injection in flies carrying TEV-sensitive cohesin complexes (Fig. 5b).

Fluorescence Recovery After Photobleaching (FRAP) 14m

29

FRAP studies on chromatin-binding proteins revealed that many have a very dynamic behavior, with turnover within seconds (e.g., transcription factors [29]). In contrast, both cohesin and condensin complexes were shown to display a slow turnover or be stably bound to mitotic chromatin [19, 20, 22]. More importantly, turnover rates may also vary for specific time points of cell cycle. Thus, microinjection techniques can be used to arrest the fast embryonic cycles at specific stages, thereby enabling long-term FRAP experiments. What follows below is an example of analysis of condensin I turnover on mitotic chromosomes, similar to the one previously published [22]. For this analysis, strains expressing fluorescent-tagged versions of the protein of interest are required.

30

Use your reference channel (e.g., fluorescent histones) to select an embryo with the required nuclear density and in late-interphase using a 63× or 100× lens.

31

Switch to a lower magnification lens for injections.

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32 Inject [M]12 mg/ml – [M]30 mg/ml UbcH10^{C114S} (intact spindle forces) or [M]2 Milimolar (mM) colchicine (microtubule poison) into the embryo to induce a metaphase arrest, if required. After 00:06:00 – 00:08:00 , every nucleus should be arrested in prometaphase or metaphase. 14m

Depending on the anticipated time of recovery, FRAP analysis can be performed in cycling embryos instead of inducing a metaphase arrest.

33 Select a field for imaging, preferably including the nuclei closer to the coverslip.

34 2m

Image for a short period a time (e.g., 00:02:00) before inducing a bleaching pulse. This will provide a reference for basal fluorescent intensity before bleaching and recovery occur.

For FRAP analysis it is crucial to minimize photodamage stimulated by laser/light power. Time between frames should also be short enough to detect fast exchange events but without further enhancing phototoxicity (e.g., 30 s/frame).

35 According to the imaging software available to induce FRAP, draw ROIs of the nuclei to be bleached, bleaching a maximum of one-fourth of the metaphases in the field (see Fig. 6a).

Several options can be used for the shape and size ROI to be bleached: bleaching of entire metaphases, bleaching of half metaphase, or bleaching of a smaller area within the metaphase (e.g., a small circle, rectangle). Be aware that recovery dynamics may be challenged by larger areas simply due to bleaching of a higher number of fluorescent molecules. Also, nuclei, although arrested at metaphase, will not be static, and may hinder FRAP efficiency. We find it best to bleach half of a metaphase plate, where the other unbleached half can be used as an internal control.

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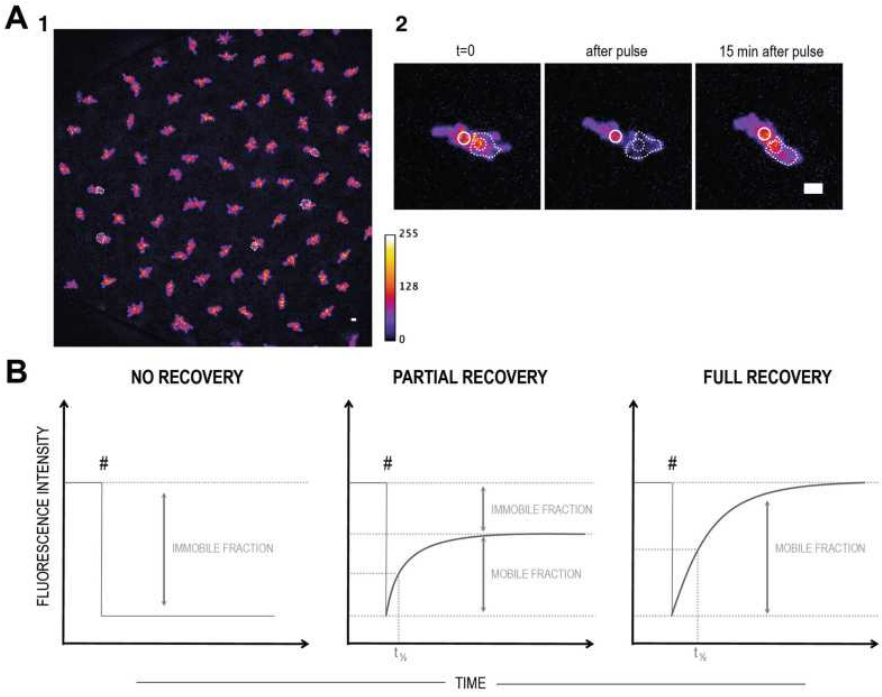

Fig. 6 Example of a typical FRAP experiment with Barren-EGFP expressing embryos.
(**a**) (1) Still image of a UAS-Barren-EGFP embryo [22] arrested at metaphase with 12 mg/ml of UbcH10^{C114S}.
(**a**) (2) Close-up of representative still images of a metaphase plate during a FRAP experiment. Fire LUT was used to emphasize a photobleaching event and subsequent recovery of fluorescence intensity. Dashed areas indicate the half-metaphase plate where it was induced a bleaching pulse. Fluorescence intensity can be measured using a small circular ROI from bleached half metaphase plate—dashed circle, and controlled with the corresponding unbleached half metaphase plate—full circle. Live imaging was performed using a confocal spinning disk microscope with MetaMorph acquisition software, using a 63× immersion (oil) objective. Time-lapse series were processed using Fiji Scale bar: 2 µm.
(**b**) Three possible scenarios can arise from a FRAP experiment: (1) after bleaching pulse, no fluorescence recovery is detected, hence, no turnover is deduced; (2) fluorescence intensity increases after pulse but not similar to prepulse intensities, indicating there was some exchange of molecules; (3) full recovery of fluorescence intensity to similar levels as before pulse, suggestive of a highly dynamic turnover. # shows the time of bleaching pulse. The difference between the plateau and *y*0 indicates the mobile fraction. *t*(time) to which half of plateau's fluorescence intensity corresponds is the half-time

36 Induce the pulse.

When optimizing bleaching pulses, take care that they should be short, typically less than 20 ms, in order to avoid phototoxicity for the sample and localized heating of the sample, generated by high laser intensity [34]. As such, increasing the laser power works better than increasing the timing of the bleaching pulse. This is also useful to minimize the diffusion effect of fluorescent molecules during the pulse [18].

37 15m

Image immediately after bleaching for a longer period of time (e.g., **00:15:00**), keeping the same settings as prebleaching imaging.

38

Analyze the recovery using quantitative imaging software (e.g., Fiji [30]).

39 The mean fluorescence intensity can be normalized in several ways (e.g., to the first time point before pulse (t0) or to

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unbleached half metaphase for each time point).

Several options can be used for the shape and size ROI to be bleached: bleaching of entire metaphases, bleaching of half metaphase, or bleaching of a smaller area within the metaphase (e.g., a small circle, rectangle). Be aware that recovery dynamics may be challenged by larger areas simply due to bleaching of a higher number of fluorescent molecules. Also, nuclei, although arrested at metaphase, will not be static, and may hinder FRAP efficiency. We find it best to bleach half of a metaphase plate, where the other unbleached half can be used as an internal control.

40

Plot the relative mean fluorescence intensity versus time in a *xy* manner.

41

For estimation of protein turnover, fit the data to the appropriate function (e.g., the One Phase Association equation $y = yo + (Plateau - yo) * (1 - exp(-K \times x))$) can be used to estimate several dynamic parameters, as indicated in Table 1).

A	B
Variable	**Definition**
Y0	Value of y at t = 0 Expressed in the same units of y
Plateau	Value that y tends to for infinite of x Expressed in the same units of y
K	Rate constant Expressed in –t (inverse of x units)
Tau	Time constant Expressed in the inverse of y units
Half-time	Time of fluorescence recovery after the pulse where the fluorescence intensity is half of the final recovered intensity Expressed in the same units of x
Span (mobile fraction)	Difference in intensity between y0 and plateau Expressed in the same units of y

Table 1
Quantitative variables from one-phase association curve fitting

42

FRAP will result in three possible scenarios: no recovery of fluorescence intensity, indicating that there was no replacement of fluorescent molecules and hence, the protein is stable and did not turn over; partial recovery of fluorescence intensity or complete recovery of fluorescence intensity, where there was limited or complete exchange of the tagged protein (Fig. 6b) [31].

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