Chapter 15

Rapid Ovary Mass-Isolation (ROMi) to Obtain Large Quantities of *Drosophila* Egg Chambers for Fluorescent In Situ Hybridization

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Abstract

Isolation of large quantities of tissue from organisms is essential for many techniques such as genome-wide screens and biochemistry. However, obtaining large quantities of tissues or cells is often the rate-limiting step when working in vivo. Here, we present a rapid method that allows the isolation of intact, single egg chambers at various developmental stages from ovaries of adult female *Drosophila* flies. The isolated egg chambers are amenable for a variety of procedures such as fluorescent in situ hybridization, RNA isolation, extract preparation, or immunostaining. Isolation of egg chambers from adult flies can be completed in 5 min and results, depending on the input amount of flies, in several milliliters of material. The isolated egg chambers are then further processed depending on the exact requirements of the subsequent application. We describe high-throughput in situ hybridization in 96-well plates as example application for the mass-isolated egg chambers.

Key words Ovary, Egg chamber, Mass isolation, FISH, *Drosophila*, Rapid ovary mass-isolation, ROMi

1 Introduction

Biological research aims towards understanding proteins, cells, tissues, and animals by observing, recording, or manipulating them ideally in their native in vivo state to test hypotheses. Isolation of high-quality material that can serve as an in vivo model for many cell biological, biochemical, and developmental questions is therefore key for many applications and often presents a bottleneck for analytical or high-throughput analyses. The isolation of material without the need for laborious micro-dissection has therefore many applications in biological research.

The *Drosophila* ovary is a key model to study cell biological, developmental, and cell cycle questions but also allows insights into mechanisms of post-transcriptional gene regulation [1–5]. Adult female *Drosophila* flies have paired ovaries that each is organized into chain-like ovarioles that harbor egg chambers of all develop-

Christian Dahmann (ed.), *Drosophila: Methods and Protocols*, Methods in Molecular Biology, vol. 1478,
DOI 10.1007/978-1-4939-6371-3_15,

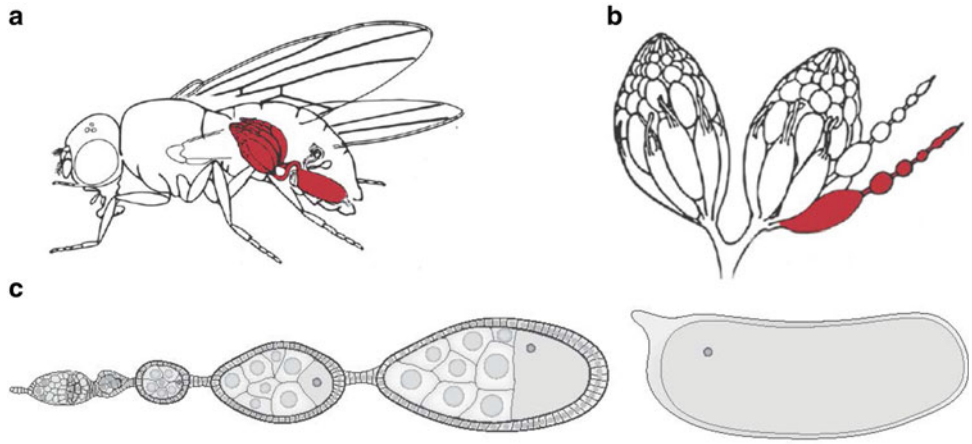

Fig. 1 Schematic representation of an ovary in a female *Drosophila* fly (**a**), of an isolated ovary with one ovariole highlighted in *red* (**b**), and organization of egg chambers in an ovariole (**c**)

mental stages (Fig. 1a, b). Each egg chamber is composed of somatic epithelial cells that overlay the germline cells, the oocyte, and the accompanying nurse cells (Fig. 1c). To obtain ovary material, the abdomen must be opened with dissection forceps, the ovaries pulled out, and remaining abdominal cuticle removed. For the analysis of single egg chambers or for whole-mount staining procedures it is essential that the muscle sheet covering the ovary is removed, ovaries are separated into single ovarioles, and individual egg chambers are isolated from the chain-like ovarioles. All these steps, opening of the abdomen and the ovary and obtaining single egg chambers, are typically done manually while inspecting the material under the microscope.

The goal was therefore to develop a protocol that would allow us to rapidly obtain large quantities of intact but individualized egg chambers that are amenable to total RNA isolation and genome-wide fluorescent in situ hybridization that typically required egg chambers from ~100,000 adult female flies. In particular the separation of the ovary into ovarioles and separate egg chambers is essential for whole-mount staining, mounting of the samples on a microscopic slide, and rapid inspection and imaging. Previous protocols have focused on isolation of stage-specific egg chambers primarily for biochemical analyses [6, 7]. Here we introduce an alternative method that is based on a kitchen-type grinding mill instead of a blender to isolate ovaries from the adult *Drosophila* abdomen that we call **r**apid **o**vary **m**ass **i**solation (ROMi). The advantage of the grinding mill is that it produces homogeneous and intact samples. It reduces the tissue loss due to damaged egg chambers and circumvents lengthy steps involving separation of egg chambers by gravity. We also describe in detail how we used the ROMi egg chambers for subsequent isolation of total RNA

and for genome-wide fluorescent in situ hybridization (FISH) screen to identify localized mRNAs [8]. For in situ hybridization we adapted a previous method for *Drosophila* embryos [9, 10] and performed the experiments in a 96-well plate format. Our protocol can be easily adapted to other types of *Drosophila* tissue such as embryo imaginal discs, testis, and even other specimen. Taken together, we have developed ROMi, a rapid ovary mass isolation, that produces (1) intact but singularized egg chambers in large quantities, (2) permits the egg chambers to be used for multiple purposes such as whole-mount staining, and (3) can be completed rapidly in five minutes.

2 Materials

2.1 Rapid Ovary Mass Isolation

1. Beaker to collect flies in, e.g., embryo collection cage (*see* **Note 1**).
2. Fixative solution: 4% Formaldehyde in PBS or PBST buffer. PBS buffer composition: 2.2.3. PBST buffer composition: 2.2.4.
3. Grinding mill (e.g., Kitchen Aid Classic Series Tilt-Head Stand Mixer with All Metal Grain Mill (Kitchen Aid, St. Joseph, MI, USA))
4. Metal Sieves (**8″ Ø × 2″ height**) 850, 425, 212 μm mesh size.
5. Embryo collection cages with stainless steel mesh—97 μm.

2.2 96-Well Fluorescent In Situ Hybridization

1. 96-Well MultiScreen$_{HTS}$ DV Filter Plates, 0.65 μm.
2. Aspirator for solutions: Eppendorf Perfect Vac Manifold Quad.
3. *PBS*: NaCl—8 g, KCl—0.2 g, KH_2PO_4—0.24 g, $Na_2HPO_4 \cdot 7H_2O$—2.72 g.

   Dissolve in 0.8 l H_2O, adjust pH to 7.4 with HCl, and volume to 1 l.

   PBT: Add 1 ml Tween-20 to 1 l of PBS.
4. 20×SSC: NaCl 87.7 g, sodium citrate 44.1 g. Dissolve in 0.4 l H_2O, adjust pH to 7.0 with 10 N NaOH, and adjust volume to 0.5 l.
5. Hybridization buffer (Hyb buffer): H_2O 150 ml, formamide 250 ml, 20× SSC 100 ml, Tween 20 0.5 ml. Total 500 ml.
6. Hybridization buffer with dextran sulfate (Hyb-Dextran): DEPC-treated H_2O 100 ml, formamide 250 ml, 20× SSC 100 ml, Tween 20 0.5 ml, 50% dextran sulfate 50 ml. Total 500 ml. For dextran sulfate: prepare 100 g of dextran sulfate in DEPC H_2O and adjust volume to 200 ml. Store at 4 °C.
7. Wash buffer: H_2O 200 ml, formamide 250 ml, 20× SSC 50 ml, Tween 20 0.5 ml. Total 500 ml.

8. Mounting solution: 20 mM Tris–HCl pH 8.0, 0.5% *N*-propyl gallate, 90% glycerol.
9. Anti-digoxigenin-POD Fab fragments.
10. Cy3-reagent.
11. 4′,6-Diamidine-2-phenylindol (DAPI) was used at 1:1000 dilution.
12. Mounting medium—100% glycerol with 2% *N*-propyl-gallate.

3 Methods

3.1 Rapid Ovary Mass Isolation

1. Flies were raised on standard laboratory conditions (*see* **Note 2**). Prior to egg-chamber isolation, mixed-sex flies were fed with a fresh suspension of active, dry baker's yeast (*see* **Note 3**). To obtain a mixture of egg chambers of all developmental stages, we mixed batches of flies fed with fresh yeast for 2 days at 21 °C and for 1 day at 25 °C (*see* **Note 4**).
2. Flies were narcotized with CO_2, collected in a beaker, and kept narcotized for a maximum of 5 min before proceeding (Fig. 2a and *see* **Note 5**).
3. To prepare flies for whole-mount fluorescent in situ hybridization, narcotized flies were immediately immersed in 4% formaldehyde in PBS (*see* **Note 6**). *Alternatively*, for the preparation of ovarian extract, for biochemical analyses, or isolation of total RNA from egg chambers, flies were immersed in ice-cold PBS instead.
4. Collected flies were rapidly processed twice through a metal grain mill adaptor for a standard food processor and the entire flow-through collected (Fig. 2b) (*see* **Note 7**). The grinding settings can vary from fine to coarse; we used a fine setting (grade step "3") (*see* **Note 8**).
5. The ground flies were size-separated using 850, 425, and 212 μm sieves successively (*see* **Note 9**) and collected in an embryo collection cage (mesh size 97 μm) that was placed in a large glass beaker (Fig. 2c, d) (*see* **Note 10**). The flow-through was highly enriched for individual egg chambers of all developmental stages (Figs. 2f and 3a). To ensure that all egg chambers passed the mesh, the sieves were briefly rinsed with a little amount of PBS (e.g., from a squeeze bottle, *see* **Note 9**).
6. Collection of mass-isolated material (Fig. 2) varied depending on the subsequent application:
   (a) For *whole-mount fluorescent in situ hybridization experiments* the co-isolation of testis and gut materials did not disturb the subsequent analysis (*see* **Note 11**). The filtrated material was kept in the embryo collection cage and

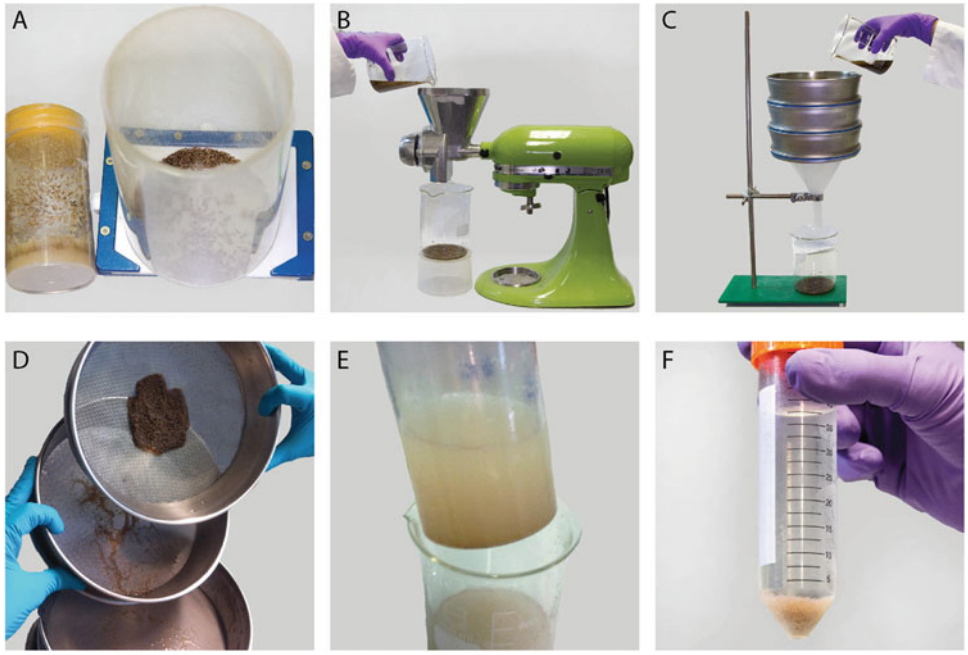

Fig. 2 Steps of rapid ovary mass isolation from whole flies to isolated egg chambers in a reaction tube. (**a**) Anesthetized flies in the collection cage on a fly pad. (**b**) Grinding of flies in a kitchen-type grinding mill. (**c**) Separation of the ovary material by sieving the ground flies. (**d**) Close-up view of the fractions retained in each level of the sieve pyramid. (**e**) Repurposed inverted embryo collection cage used to wash the ovary material after isolation. (**f**) Isolated ovary material in a Falcon tube

remained in fixative solution for an additional 15 min, resulting in an overall fixation time of 20 min interrupted by occasional stirring of the material. The cage with egg chambers was then transferred to a new beaker containing PBS and washed twice for 5 min, replacing the PBS solution after the first round. The egg chambers in the embryo collection cage were then transferred stepwise into 100% methanol in the following sequence: 25% methanol in PBS, 50% methanol in PBS, and 75% methanol in PBS. At each step egg-chambers were equilibrated for 5–10 min with occasional swirling of the beaker. Finally, egg chambers were removed from the cage/sieve by gently tipping them into a 50 ml reaction tube and releasing egg chambers from the bottom of the sieve using a methanol-filled squeeze bottle (*see* **Note 12**). The washing methanol was replaced with fresh methanol after allowing the egg chambers to settle by gravity (~5 min). The egg chambers were then used for whole-mount in situ hybridization (*see* Subheading 3.2) or stored at −20 °C (*see* **Note 13**).

(b) For *isolation of total RNA* we kept the mass-isolated egg chambers on ice in cold PBS and manually selected

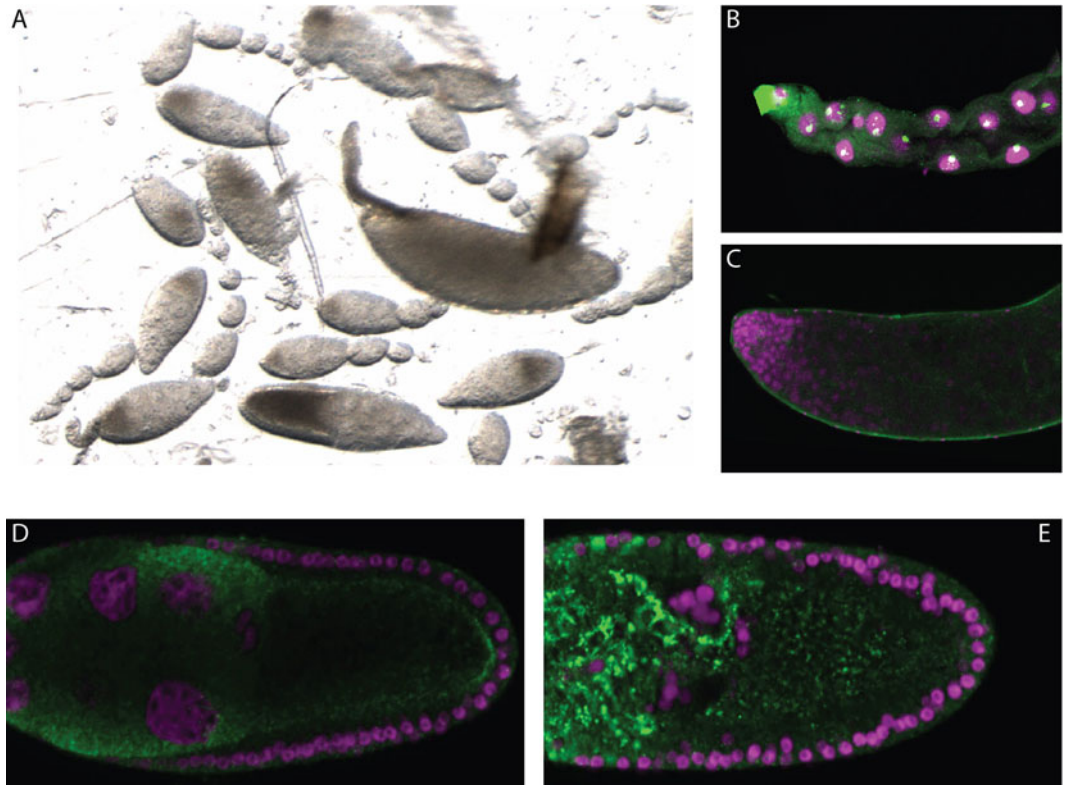

Fig. 3 Image of egg chambers isolated by mass isolation (**a**). Fluorescent signal in gut (**b**) and testis (**c**) tissue "contaminations" after in situ hybridization. Properly fixed mass-isolated egg chambers after fluorescent in situ hybridization against *mapmodulin* mRNA (**d**) compared with material fixed only after isolation (**e**)

egg chambers at early stages (germanium to stage 7, previtellogenesis), late stages (stage 9–10, postvitellogenesis), and full ovaries highly enriched for stage 11+ egg chambers using a stereomicroscope. For each stage we collected at least 10 μl of total material that was snap-frozen immediately.

(c) For preparation of *ovarian extract*, mass-isolated egg chambers were collected in a 50 ml reaction tube and briefly spun down (e.g., at 1000 rpm in a table-top megafuge) and any excess PBS supernatant removed. This yielded a highly concentrated egg-chamber extract useful for example for in vitro translation reactions [11].

3.2 96-Well Fluorescent In Situ Hybridization

1. Mass-isolated egg chambers were transferred stepwise into PBT in a suitable reaction tube (*see* **Note 14**). Each step was allowed 5-min washing time on a rotating table and ~5 min

to settle by gravity. The following solutions were used sequentially: 75 % methanol in PBT, 50 % methanol in PBT, and 25 % methanol in PBT.

2. Egg chambers were then washed six times in PBT to remove any methanol, each wash step again taking 5 min on a rotating table and another 5 min to allow egg chambers to settle by gravity.
3. Egg chambers were then briefly washed in a solution of PBT and Hyb (1:1) before being pre-hybridized in 100 % Hyb for 1 h at 55 °C.
4. Egg chambers in Hyb solution were then transferred to a 96-well plate by using a pipette tip with a wide opening that prevents material from getting stuck. Hyb solution was then removed by vacuum and replaced with 200 µl of Hyb-dextran buffer containing the antisense RNA probes (*see* **Note 15**). Hybridization of RNA probes was allowed overnight at 55 °C on a rocking platform (*see* **Note 16**).
5. The next day 100 µl of pre-warmed (55 °C) wash buffer was added to each well and immediately removed together with probe solution by vacuum.
6. Egg chambers were once more rinsed with 150 µl of wash buffer and then washed four times for 1 h each in 150 µl of wash buffer at 55 °C.
7. We next washed egg chambers five times for 1 h each at 55 °C in 150 µl PBT; the last wash was left overnight at 55 °C (*see* **Note 17**).
8. The next day, egg chambers were washed twice for 1 h in 150 µl PBT at room temperature.
9. After the last wash, 200 µl antibody solution was added per well and incubated overnight at room temperature. The antibody solution contained anti-DIG antibodies diluted 1:200 in PBT.
10. On the last day, egg chambers were rinsed with 150 µl of PBT and then washed ten times in 150 µl of PBT for 30 min at room temperature (*see* **Note 18**).
11. For detection egg chambers were incubated with Cy3-tyramides 1:70 diluted in 50 µl of amplification buffer for 30 min.
12. Egg chambers were then washed ten times for 30 min at room temperature in 150 µl of PBT. DAPI, diluted 1:1000, was included in one of the wash steps (*see* **Note 19**).
13. Finally, all PBT was removed and ~100 µl mounting medium was added (*see* **Note 20**).

4 Notes

1. To collect adult flies we used an embryo collection cage that has a perforated bottom (stainless steel mesh) that we placed on a fly pad. This way, the flies were kept narcotized until enough were collected.
2. For standard laboratory conditions of raising flies please refer to the webpage of the Bloomington Stock Center (http://flystocks.bio.indiana.edu/Fly_Work/culturing.htm) or standard literature on *Drosophila* as model system for example [12].
3. For the purpose of whole-mount staining or hand-selection of egg chambers for RNA isolation, the presence of gut and testis from male adult flies in the preparation is unproblematic (Fig. 3b, c). If necessary, adult males could be removed prior to mass isolation either manually or genetically using the hs-hid system that prevents eclosion of male flies [13].
4. The time and temperature at which flies were fed with fresh yeast prior to mass isolation are not critical; instead, the incubation time (1–2 days) and temperature (18–29 °C) can be experimentally determined and adjusted depending on required material. Prolonged incubation time and higher temperature simply increase developmental speed and result in enrichment of older egg chambers.
5. The beaker had a diameter of 10 cm and we collected between ~400 and up to ~4000 flies per round of mass isolation. These were between 0.3 and 3 g of fly material, but the exact weight can vary with feeding conditions and strains used. Too many flies per round of mass isolation can decrease the purity of the sample.
   Keeping flies narcotized for prolonged time periods strongly impaired the egg-chamber constitution and resulted in necrotic egg chambers.
6. Grinding the flies in the presence of fixative greatly improved the condition of the egg chambers: flies that were subjected to grinding in PBS and fixed subsequently experienced deformation of the egg chamber due to physical stress; such deformations were clearly visible in the staining procedure and can greatly influence the mRNA distribution (Fig. 3d, e). The volume of the fixative is not relevant; however we typically used 100 ml fixative for 400–4000 flies (0.3–3 g).
7. Using a grinding mill instead of a blender as described in previous protocols is critical—by using a kitchen-type grinding mill each fly will experience a similar pressure and pass the grinder only once. In a blender flies are often cut repeatedly and the site where the blade cuts is variable. Consequently, using a blender the tissue is inhomogeneous and many ovaries are damaged.

8. The grinding step size will have to be determined for the specific equipment used for the grinding. The standard Kitchen Aid grinding mill has 10 step-sizes. Counting from the finest setting, we used the third-grade step. In our hands, a finer setting destroyed the egg chambers; a coarser grinding step size left flies mostly intact.
9. Smaller mesh sizes can be used depending on the requirements. For instance to obtain only young egg chambers of stages 2–8 it is possible to remove older egg chambers using a 90 μm or even a 75 μm wide sieve. In instances, for example to prevent dehydration, it might be advantageous to perform the washing while the sieve is partially immersed in solution, e.g., for isolating material for biochemistry.
10. We repurposed embryo collection cages into a container with a mesh bottom (Fig. 2e). The mesh size was small enough to prevent egg chambers from leaking but allowed them to be immersed in solution. Using the sieve, egg chambers could be easily transferred from one solution to the next.
11. In the whole-mount staining procedure experiments are inspected by eye and non-egg chamber materials are therefore easily spotted. In some cases, the small amount of gut or testis material even provided useful information.
12. Be careful not to loose material. It is also possible to collect the egg chambers into a beaker first and then gently tip them into a 50 ml reaction tube. In case you are also using the plastic embryo collection cage, be careful to immediately wash the methanol off after removing the material; otherwise the plastic easily cracks when methanol is evaporating.
13. We have stored our egg chambers in methanol at −20 °C for up to 2 years without visible damage.
14. We used approximately 1.5 ml of mass-isolated egg-chamber material for one 96-well plate. For this, a suitable container is a 15 ml reaction tube and for each washing step 12 ml of buffer was used. Whenever mass-isolated tissue immersed in methanol is being transferred by a pipette be sure to pre-wet the tips and use tips with wide opening.
15. The hybridization buffer differed from the pre-hybridization buffer solely by the presence of dextran. Of the in vitro-transcribed probes 0.5–5 ng labeled RNA per reaction was typically sufficient.
16. All washes and incubation in 96-well plates were done on rocking platform to gently mix the solution. To avoid evaporation of buffer overnight at high temperatures, seal the lid of the plate.
17. Again, to avoid evaporation of buffer overnight, seal the lid of the plate.

18. The total volume of the washes is more critical than the incubation time; if necessary shorten the washes to 10 min, but do not use less than ten washes.
19. Again, the total volume of the washes is more critical than the incubation time; if necessary shorten the washes to 10 min, but do not use less than ten washes.
20. A little bit of PBT left does not do any harm. Make sure to let the egg chamber equilibrate in mounting solution at least overnight before mounting them on a microscope slide. When transferring egg chambers from 96-well plates to microscopic slides wet the pipet tips in mounting solution, use tips with wide opening, and pipet carefully due to high viscosity of the mounting solution.

Acknowledgements

We would like to thank Daniel Lakatos and Franziska Friedrich for drawings of fly ovaries, Michaela Rupprecht for photos of ROMi procedure and help with processing the photos, and Daniel Lakatos for critical reading of the manuscript.

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