1. **Preparation of *Drosophila* females for ovary dissection**
	1. Sprinkle dry kitchen yeast on fresh standard fly food placed in small vials (ca. 15 mL). Place a 1:2 ratio of 1-2 day old males and females (max. 10 males:20 females) into the vial and let them feed for 2-3 more days to induce egg chamber production and oviposition in females.
	2. Keep these flies at room temperature (23‒25 °C) or in a 25 °C fly-specific incubator in the same vials as stated in 1.1.
	3. Flip flies into a new vial with fresh food without kitchen yeast and prepare a Schneider mix as stated in the protocol step 2.
2. **Preparation of Schneider mix for ovary dissection**

2.1. Use sterile 50 mL conical tube and add 82% Schneider Medium: Add 17% Fetal Bovine Serum > Add 0.6% Streptomycin/Penicillin. Mix this Schneider mix (SM) well.

2.2. Remove small particles by using a filter (see Table of Materials). Adjust pH = 6.95-7.00 of the SM with 1N HCl. Store the SM at 4 °C for maximally 14 days.

* 1. Before dissection of *Drosophila* ovaries, add insulin to the SM to prepare fresh Schneider Medium with Insulin (SMI). The final insulin concentration should be 0.2 mg/mL. Allow the SMI to reach room temperature.

NOTE: Add insulin to the SM just before planned dissection. Never use older SMI than 8h stored at 4 °C.

* 1. Pipette 100 µL of the room-tempered SMI onto the prepared depression dissection glass and 100 µL onto the glass part of a dish at the dissection stereoscope.
1. **Dissection of *Drosophila* ovaries**

3.1. Anesthetize flies in the prepared vial from the protocol step 1 and place them onto a CO2 supplied fly pad to immobilize them. Under the dissection stereoscope, select 4‒6 females and leave them on the fly pad.

* 1. Use forceps to place one anesthetized female from the fly pad and place onto the SMI in the depression dissection glass.
	2. Under the dissection binoculars (ca. 2x magnification) provided with a cold light, hold the female by the thorax and the adjunct part of the abdomen with one of the forceps and pull the abdomen cuticle (the dorsal part that is closer to the end tip of the abdomen) with the other forceps in the opposite direction.

NOTE: In case that the whole abdomen separates from the rest of the female body (thorax), discard the abdomen and dissect another female. However, once experienced with dissection of the ovaries, it is easy to identify and dissect ovaries also from the separated abdomen.

* 1. Ovaries will be pulled off with the other fly organs placed in the abdomen. Select ovaries, they look like two bundles of grapes and place them into the freshly prepared SMI on the dish (see the **Table of Materials**).
	2. Hold gently the widest part of ovaries with one pair of forceps and hold one of the oldest egg chambers with the other forceps at this part. Pull out gently the oldest egg chamber out of ovaries. The string of egg chambers called ovariole should be automatically pulled out together with the oldest egg chamber. Should it not be the case, repeat this step with a slightly younger staged egg chambers.

NOTE: Pull always on the other side than egg chambers of your interest are placed in ovaries.

* 1. As ovarioles are covered with a contractile muscle sheet, check that dissected ovarioles are properly separated from the tip of ovaries and other ovarioles to ensure that muscle sheet was removed.

NOTE: Do not touch egg chambers of your interest with forceps to avoid damaging them.

* 1. Select six to seven separated ovarioles, which appear as a node-free string. By using the cactus tool, move them gently in the SMI to one side of the dish. Chose a side where a minimum of debris and old egg chambers are placed*.*

NOTE: If individual egg chambers of the interest (stages 6‒8) are present in the SMI, include them too.

* 1. By using forceps, remove then all unwanted egg chambers together with debris, a dissection product from the SMI.

NOTE*:* If the SMI still appears dirty, pipette 2/3 of the SMI in the dish gently out. Do it on the other side than the egg chambers and ovarioles of your interest are placed in the dish. It is crucial to prevent egg chambers from drying out.

* 1. To visualize cell membranes in egg chambers, add 1 µL of the 1:100 membrane dye to the 100 µL SMI with the selected ovarioles in the dish about 10‒15 mins before planned imaging.

NOTE*:* This estimated time is essential to allow the dye to penetrate into the egg chamber tissue. Optimization may be required for all other applications.

* 1. Mix gently with dissecting forceps. Avoid contact of ovarioles with the forceps though and cover the dish by a lid provided by the manufacturer.
1. **In vitro life imaging of actomyosin signals at the local cellular scale (up to 15 analyzed cells per an egg chamber)**

NOTE: This protocol step requires an inverted confocal microscope with a 63x water objective to obtain sufficient resolution of actomyosin signals, corresponding laser lines to image fluorescent tags/dyes, a camera to acquire time-lapse movies (TLMs) in various channels and a standard computer storage (up to ca. 70 MB per one TLM).

* 1. Immobilize cultured egg chambers and ovarioles.
		1. Take the closed dish with six to seven ovarioles and free egg chambers to an inverted confocal microscope. Gently attach the dish to the specialized microscope holder that is designed for Petri dishes.
		2. Open the lid and spread the selected ovarioles or older individual egg chambers (stages 6‒8) with a cactus tool under 10x magnification and differential interference contrast (DIC) microscopic set up. Let the ovarioles settle down and make sure that they are substantially far from each other to avoid any potential motion generated by neighboring ovarioles.
		3. Control egg chambers and ovarioles for rests of the muscle sheet and remove all ovarioles with muscle sheet from the SMI. Close the dish with its provided lid to avoid drying during microscopy session.

NOTE: Muscle sheet that covers egg chambers in ovarioles is contractile and a source of a potential undesirable movements of ovarioles. This is a crucial step as ovariole movements may lead to the focus loss during imaging.

* 1. Acquisition of TLMs for detailed actomyosin behavior
		1. Focus the microscope with 10x objective and DIC on one selected ovariole and inspect again for presence of muscle sheet.
		2. Find an egg chamber of the interest in the selected muscle-sheet-free ovariole. By using DIC settings, focus microscope into the middle of this egg chamber.
		3. By using fluorescent reflectors, check for the appearance of cell membranes in the selected egg chamber, any strong dye staining point to a damage of the egg chamber. Do not image this egg chamber and search for other healthy one.
		4. Set up acquisition parameters including corresponding laser lines, laser blocking filters, camera, etc. on the microscope. Follow providers instructions.

NOTE: Avoid strong laser power as it can damage/burn egg chambers. The damage of egg chambers can be observed as a sudden absence of epithelial rotation and in the worst case as a visible tissue damage during TLM acquisition. The correct settings must be tested in advance prior to TLM acquisition of selected egg chambers.

* + 1. Change the 10x to a water 63x objective with numerical aperture (NA) of at least 1.3. Use water immersion to keep the light refraction similar to the used medium.

NOTE: A 63x objective provides sufficient resolution to observe subcellular actomyosin signals in egg chambers. Other objectives with lower resolution and lower NA will not give clear subcellular actomyosin signal that can be later comfortably analyzed.

* + 1. Focus on the middle central area of the egg chamber and then on the most outer circumferential area of the egg chamber. Slightly lower the focus onto the surface of egg chambers with clear actomyosin signals.
		2. Set up confocal imaging for single plane scans with time intervals of 6‒12 s for 5‒10 min to achieve short-time high-speed live imaging of the tissue surface of a selected egg chamber.

NOTE: No *z*-stack is required to acquire outer circumferential actomyosin behavior for ca. 5 cells in egg chambers of stage 1‒5 and for up to 15 cells in egg chambers of stages 6‒8. Before using the microscope, make sure that the microscopic table is well sprung/air-cushioned from the floor to reduce vibration that could affect imaging.

* + 1. Save the desired TLMs.

NOTE: All file formats that can be opened as a time-lapse stack in Fiji (e.g., through **Bioformats**) can be used.

* + 1. Note the orientation of a TLM, i.e., where is the anterior and posterior side of the imaged egg chamber. This information is required for further analysis.

NOTE: After imaging of one egg chamber, follow directly an acquisition of a TLM of another independent egg chamber in the same dish. With practice it should be possible to image up to 10 individual egg chambers of stages 1‒8 (i.e., 10 acquired TLMs) within one imaging session of 2 h.