**Barbara Fasulo and William Sullivan**

# Live Analysis of precisely timed drug injections in the *Drosophila* embryo.

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## 1. INTRODUCTION

Several technical advances have enabled the observation of cellular processes in living organisms. The development of laser scanning confocal microscopy provides a powerful means for the observation of structures and components within a cell. Significant advances have also been made in the development of techniques for generating functional fluorescently labeled proteins. Both these tools enable the study of specific aspects of the cell cycle with high temporal and spatial resolution.

By means of confocal microscopy, it is thus possible to observe the dynamics of a particular fluorescently tagged protein in the context of a wild type cell. In addition, this same fluorescently tagged protein can be observed in a mutant background, allowing its role to be determined in a given process.

The model organism *Drosophila melanogaster* is particularly well suited for live image analysis. In *Drosophila*, the events of early embryogenesis have been characterized in detail using both fixed and live analysis. The initial nuclear divisions are rapid (10-20 minutes long), synchronous, and syncytial. The embryo carries out 13 cycles of nuclear division and DNA replication until gastrulation. Between nuclear cycles 8 and 10, the majority of the nuclei migrate to the cortex where they form a syncytial blastoderm that undergoes four more rounds of nearly synchronous mitotic cycles. At this point, a large number of nuclei are clearly visible, and can be followed simultaneously.

The duration of S phase increases progressively in each of these divisions. These syncytial divisions alternate between M and S phases with no obvious G1 and G2 phases, while development is maintained and regulated by maternal products. These synchronous divisions provide an excellent system in which to study the effects of drugs on the mitotic cycle.

The availability of GFP-histone-bearing flies and rhodamine-labeled tubulin, in conjunction with confocal microscopy, allow us to portray different stages of the cell cycle, in real time. In other words, it is possible to follow the progression of multiple nuclear and cytoskeletal events of the cell cycle, such as nuclear envelope formation, nuclear envelope breakdown, spindle formation, chromosome condensation, and segregation. Figure 1 illustrates the power of this approach. Panel A shows nuclear envelope formation (NEF), which occurs at the end of telophase and is characterized by the exclusion of tubulin (in red) from the nuclei (in green). As shown in panel B, the appearance of bright histone-GFP dots in the nuclei marks the initiation of chromosome condensation (ICC) while, in panel C, more defined and condensed chromosomes pulling away from the nuclear envelope define a second phase of condensation (CC2). Nuclear envelope breakdown (NEB) is characterized by entry of tubulin in the nuclear space (D). Panel E shows metaphase (MET), achieved by the formation of a bipolar spindle and alignment of condensed chromosomes on the metaphase plate. In panel F, elongation of the metaphase spindle, in addition to segregation of chromosomes, indicates the initiation of anaphase (IA).

This analysis permits a detailed morphological and temporal description of nuclear and cytoskeletal events in normal, untreated embryos or in drug-treated embryos. It is therefore possible to identify drugs that, when injected into the embryo, do not show any visible phenotype but do alter the relative timing of cell cycle events. Furthermore, given that we can follow, in real time, the series of events that take place in the embryo, it is

possible to choose the appropriate time of injection of a particular drug. For example, if we want to time the S phase in a colchicine-injected embryo, it is necessary to inject the drug after anaphase when chromosomes have already separated and there is no risk of activation the spindle checkpoint.

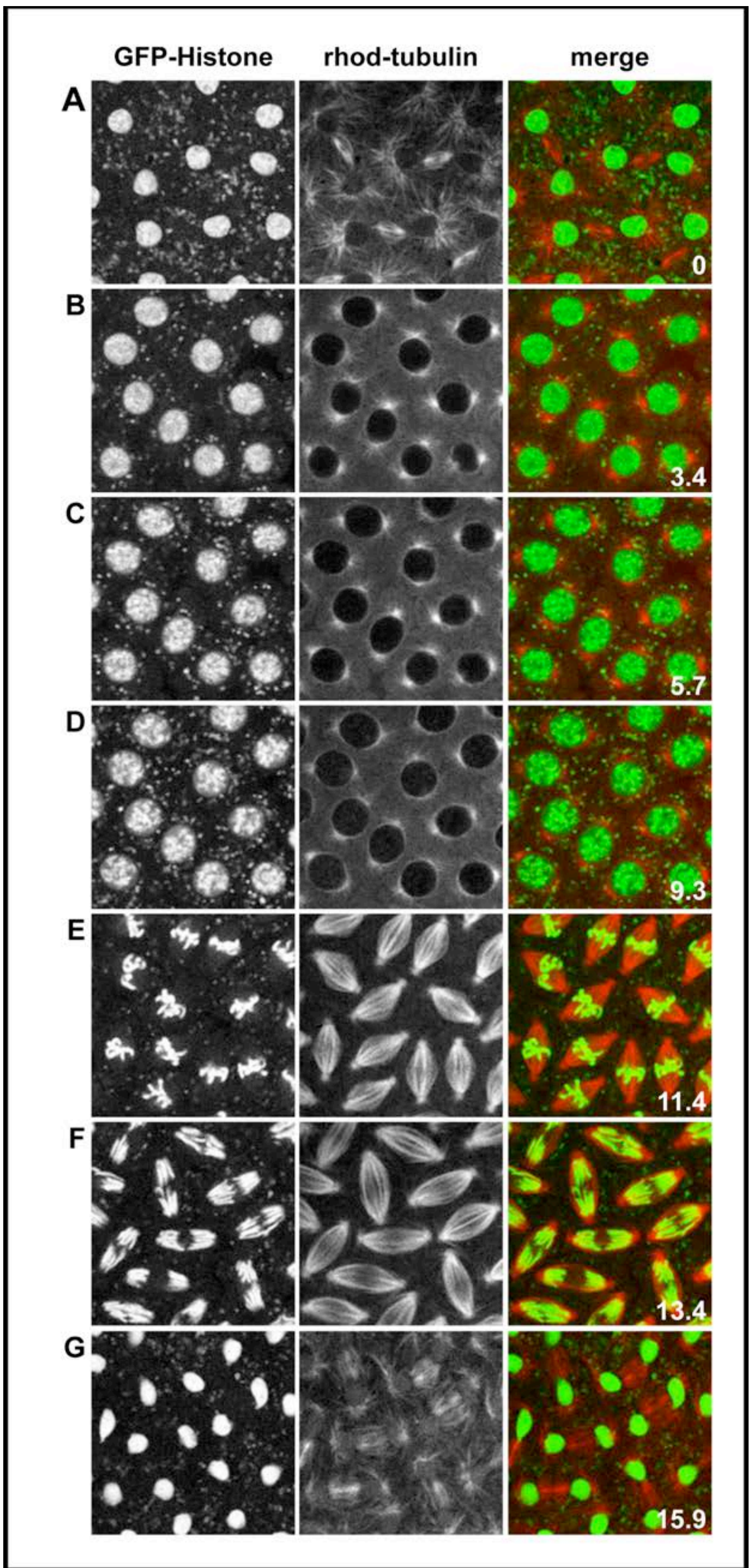


Figure 1

## 2. MATERIALS

### 2.1 Embryo preparation

1. Mechanical needle puller.
2. Injection apparatus.
3. 75 mm capillary tubing with an outer and inner diameter of 1.21 and 0.90 mm respectively (Drummond Scientific Broomall, PA cat. no. N-51 A).
4. Atoxic clay mount in a Petri dish.
5. 10 µl Hamilton syringe.
6. Microscope slides (Fisher).
7. 22 X 50 $mm^2$ coverslips (Fisher).
8. 22 X 22 $mm^2$ coverslips (Fisher).
9. Tape (Fisher)
10. Dissecting forceps
11. Halocarbon oil (Series 77, CAS no. 9002-83-9, Halocarbon Products Corp.).
12. Heptane.
13. Double-stick tape (3M).
14. Refrigerated microcentrifuge.
15. Drierite granular dessicant
16. Lids from 35 X 10-mm disposable plastic tissue-culture dishes
17. Plastic "flies collection" bottles

### 2.2 Visualization and drug treatment

1. Inverted microscope with confocal imaging system.
2. Fluorescently labeled tubulin: Molecular Probes (Eugene, OR), or Cytoskeleton, stored at -80°C in 2 µl aliquots of 20 µg each.
3. GFP-Histone fly stock kindly provided by Robert Saint.

## 3. METHODS

### 3.1 Preparation of glue:

To allow the embryos to remain stably attached to the coverslip during the period of observation, prepare a concentrated embryo adhesive: 10 ml of Heptane are added to a 50 ml conical tube containing 12 inches of double-stick tape and the tube is allowed to rotate overnight. The resulting solution is aliquoted in 1.5 ml microcentrifuge tubes and then centrifuged at 14000 rpm (15,800g) for 10-15 minutes to remove the particulate matter. The clear solution is then transferred to a tightly closed container to avoid evaporation. To make a working solution, the concentrated glue should be diluted roughly five times.

### 3.2 Preparation of needles

Draw out a few needles with a mechanical needle puller. Examine the tip of each needle under a dissecting microscope. It should be unbroken and smooth. Store the needles upon a clay mount in a Petri dish as shown in Figure 2 and keep them at 4 °C.

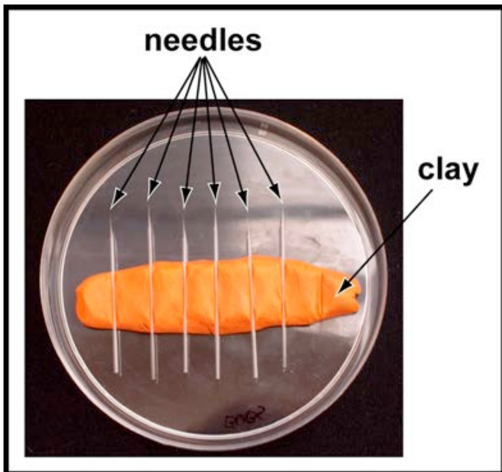

Figure 2

**3.3 Needle-slide preparation**
Using a drop of the embryo adhesive, attach a 22 X 22 $mm^2$ coverslip to the left or right side of the 22 X 50 $mm^2$ coverslip's surface (Figure 3). Store it in a place protected from dust.

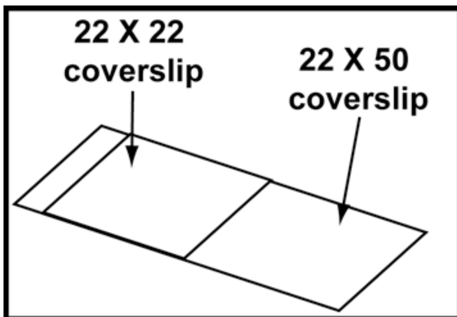

Figure 3

**3.4 Embryo slide preparation**
Attach a 22 X 50 coverslip to a slide with a piece of tape. Then attach a piece of double stick tape to one end of the slide. Pour a strip of embryo adhesive along the short axis and in the center of the slide (Figure 4). For convenience, it is useful to mark the position of the glue with a marker. Several of these slides can be prepared in advance and stored in a place protected from the dust.

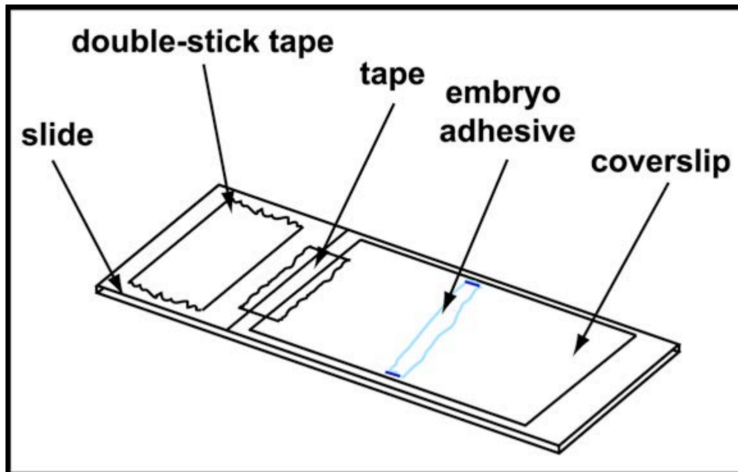


Figure 4

**3.5 Collecting embryos**
One day before "*in vivo*" analysis, place 100-200 adult *Drosophila* flies (equal numbers of male and female) in a collection bottle (Figure 5) made from a 6-ounce plastic bottle. On one side of the bottle make a hole and plug it with cotton to let the flies breathe. Spread a little yeast paste in the middle of a grape juice agar plate, and use the plate to close the collection bottle. Invert the bottle and place it at 25°C overnight. The following day, discard the old grape juice agar plate and replace it with a new one. In order to collect staged embryos, from cycles 10 to 13, let the flies lay for one hour. The grape juice plate is then removed and protected, allowing the deposited embryos to age for a further hour. Usually the first hour of collection is discarded.

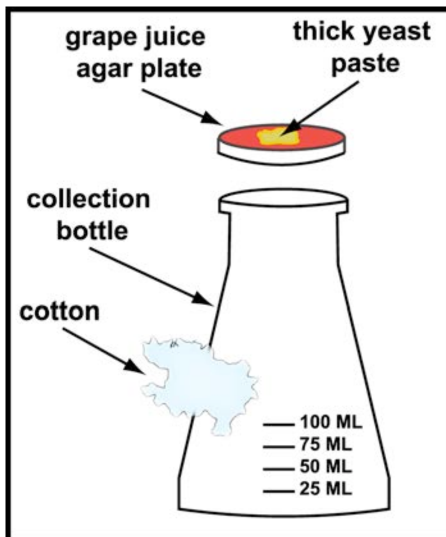


Figure 5

**3.6 Needle filling and breaking**
While the embryos age, thaw a 2 μl aliquot of rhodamine-labeled tubulin on ice and centrifuge it for 3-5 minutes at 14000 rpm in a refrigerated microcentrifuge. This pellets

denatured or aggregated protein and reduces chance of the needle clogging. Immediately after centrifugation, draw up the labeled protein with a pre-cooled Hamilton syringe, avoiding the pellet (Figure 6). Use this syringe to fill as many cold needles as possible with the labeled protein.

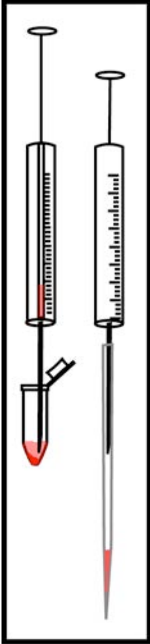

**Figure 6**

To prepare the needles with the drug, choose the appropriate dilution and follow the same procedure as above.
To break the needles, take the needle preparation slide and add a small amount of Halocarbon oil on the edge of 22 X 22 $mm^2$ coverslip (Figure 7). Place the slide on the stage of a microscope with a 10-20X objective. Mount the needle in the microinjection apparatus and apply gentle pressure. Direct the tip of the needle towards the edge of the coverslip covered with the Halocarbon oil, and break the tip by maintaining pressure. A break is promptly identified by a bubble of fluid released from the tip of the needle into the oil. Given that syncytial embryonic nuclei are 5 µm, try to generate a small hole, approximately 3-5 µm in diameter. Immediately after breaking the needles, store them upon a clay mount in a Petri dish and keep them on ice, protected from the light. A rhodamine-labeled tubulin needle can be used for up to two days if kept rigorously on ice and at 4°C over night.

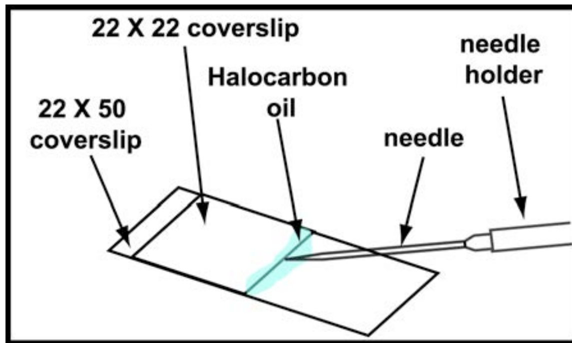


Figure 7

**3.7 Dechorionation of embryos.**
Transfer some embryos from the aged collection onto the double stick tape on the embryo slide. To dechorionate the embryos, roll them on the double stick tape and align them on the film of glue. This should be done as quickly as possible to avoid excessive dehydration. In order to prevent the flow of cytoplasm when the embryos are injected, the slide is placed in a dehydration chamber for 4 to 10 minutes. The time of dehydration depends on the day-to-day variation in temperature and humidity. The dehydration chamber consists of a Tupperware box containing Drierite granular dessicant. Once the embryos are dehydrated, they are covered with a film of Halocarbon oil to prevent further dehydration. The coverslip with the aligned embryos is then removed from the slide and is placed on the microscope stage for injection.

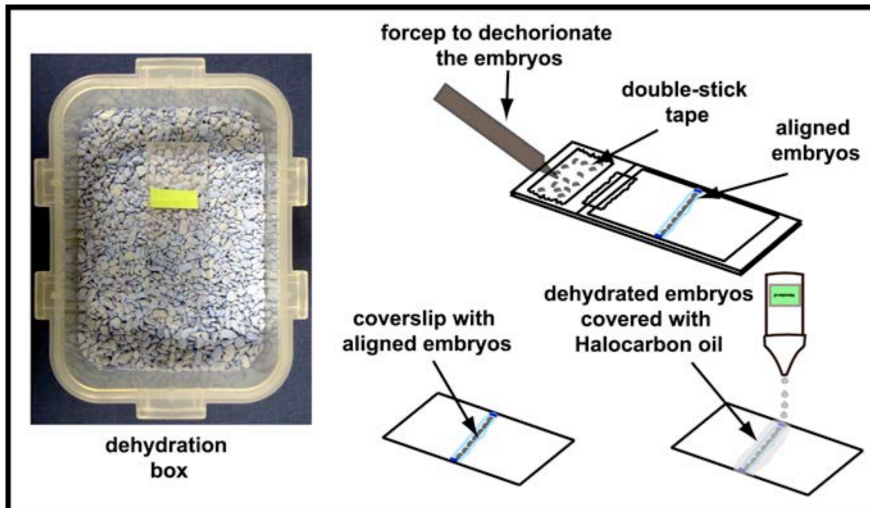


Figure 8

**3.8 Tools needed for injection**
To perform the microscopy we use an inverted photoscope (DMIRB; Leitz) equipped with a laser confocal imaging system (TCS NT; Leica).
To set the microscope for injection, a micromanipulator and a microinjector are required. The microinjector we use consists of a 20 ml siringe connected via a rubber tube to a needle holder (Figure 9).

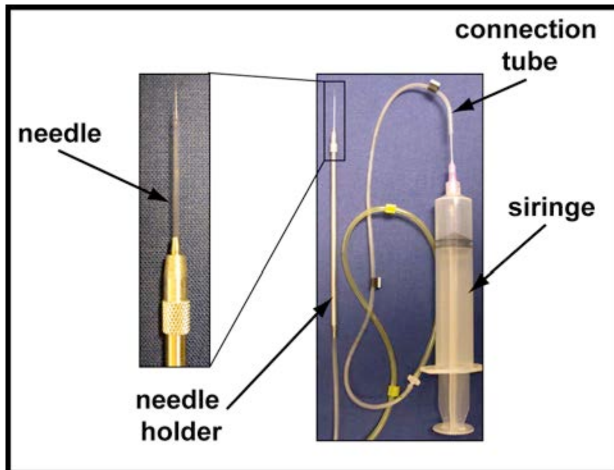


Figure 9

The micromanipulator is a Narishige Joystick Micromanipulator model MN-151. As shown in Figure 10, this allows the needle to be moved along the three axes: x, y, z, by micro-movements with knobs (red head arrows in the picture).

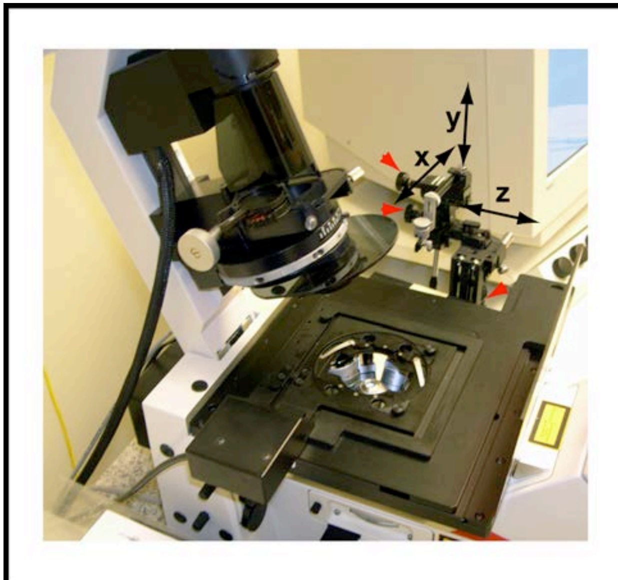


Figure 10

**3.9 Setting the microscope**

For the acquisition of images, we select a specific set of acquisition parameters. We set a two-channel recording for the two fluorescent dyes FITC and TRITC. The FITC channel is used to visualize GFP-Histone and the TRITC channel is used for rhodamine-tubulin. To observe the needle and embryo simultaneously during injection, the 20X microscope objective is more appropriate. To record the data stack we are interested in, we select the time-scan mode xyt, and 512 width and 512 height as scan format. We configure a time series with intervals not >20 sec.

### 3.10 Injecting and filming the embryo

After the embryos have been dehydrated and covered with Halocarbon oil, the 50X22 coverslip is placed on the microscope stage (Figure 11). Examining the entire row of aligned embryos, those embryos with visible germ line cells on the posterior pole are chosen. This is a visible marker which indicates the nuclei have reached the surface. To inject drugs we select embryos at cell cycle 12. By setting the acquisition parameters in continuous scan mode, it is possible to find the embryos at the right cell cycle by the number of nuclei on the surface. Furthermore, the continuous mode allows us to optimize image quality, because we can modify the scan parameters and check the results directly in the image, while the embryo is being scanned. With bleach-sensitive specimens it is advisable to restrict the use of the continuous scan function to short time intervals. Subjecting the embryo to the laser's continuous light can damage it photochemically. Once the right embryo has been visualized, the broken needle containing rhodamine-tubulin is removed from the Petri dish on ice and is placed in the needle holder. This should be done just before injection to avoid deterioration of the dye. The needle holder is then blocked on the micromanipulator. Immediately before injection, check whether the rhodamine-tubulin containing needle has clogged. If so, exchange it with another previously prepared needle.

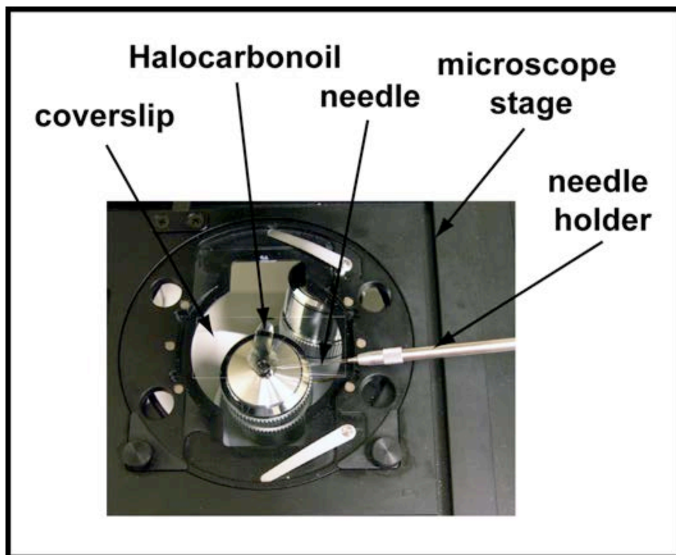


Figure 11

After the embryo is in focus, the tip of the needle is focused on the same plane and is slowly moved close to its surface as shown in Figure 12a. With resoluteness, the microscope stage with the coverslip is moved towards the needle and the tip of the needle is allowed to penetrate the embryo (Figure 12b), just below its surface. During injection we try to avoid the release of the dye between the vitelline and the plasma membrane. If the embryo is well dehydrated, the dye will be taken up without having to apply pressure with the syringe (Figure 12b). If the embryo is not sufficiently dehydrated it will reject the excess of liquid, as shown in Figure 12, c and d. The injected rhodamine-tubulin is immediately visible in the interior of the embryo, but will take almost a cycle before it is uniformly distributed. Do not inject excess amounts of rhodamine-tubulin if a second

injection is still to be performed in the same embryo, as the embryo cannot withstand large quantities of liquid. To avoid photobleaching of rhodamine-tubulin, try to inject it a short time before drug injection. Remove the needle with rhodamine-tubulin and place it on ice as soon as the embryo has been injected.

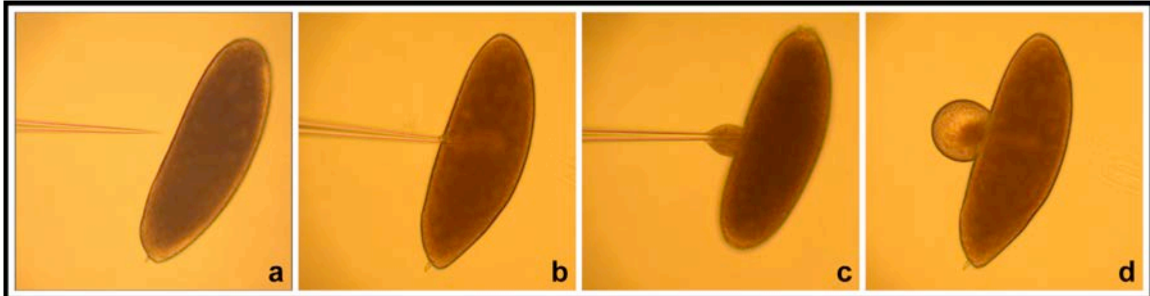


**Figure 12**

By following the dynamics of rhodamine-tubulin on the monitor (avoiding using the continuous mode for too long), it is possible to determine the appropriate time for drug injection. For example, if the aim is to inhibit DNA replication by injection of Aphidicolin, choose to inject immediately after nuclear envelope formation, when DNA replication is initiating or to time the S phase in a Colchicine-injected embryo, it is necessary to inject the drug after anaphase when chromosomes have already separated and there is no risk of activation of the spindle checkpoint. When injecting the drug, it is advisable to use the same hole created when injecting rhodamine-tubulin. The pressure applied trying to make a second hole could allow the cytoplasm to exit through the first hole.
When injecting the drug, it is advisable to use the same hole in the embryo as for the rhodamine-tubulin injection. The pressure applied trying to make a second hole could allow the cytoplasm to exit from the first hole.
In continuous mode, prior to filming the embryo, we define the acquisition parameters to optimize image quality: the exact z-position within the embryo, the amplification factor of the selected detector (for GFP or rhodamine), the diameter of the detection pinhole and the zoom factor. If the acquisition is three-dimensional (3D series), we define the upper and lower limit of the data set to be acquired and the number of optical sections. Next, we begin scanning to create the image series. During this process, it is advisable to monitor the GFP-Histone and rhodamine-tubulin signals. Photobleaching may weaken the signals, and they should therefore be adjusted accordingly. Figure 13 shows an example of an embryo injected with the S-phase inhibitor Aphidicolin. Time is shown in minutes on the right side of each picture.

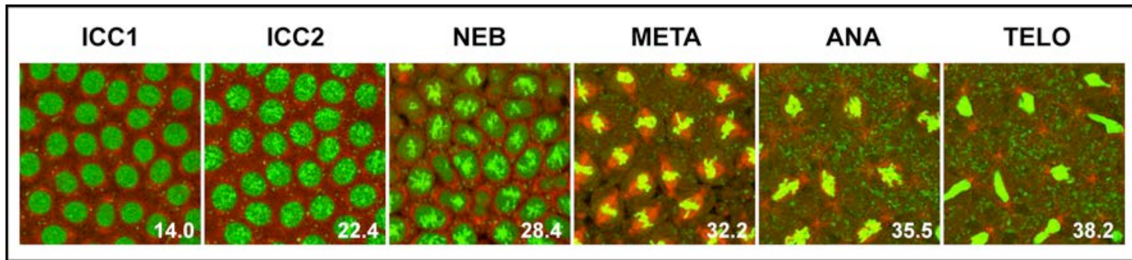


Figure 13

### 3.11 Saving data

Individual images can be saved as TIFF files and movies can be saved as AVI files.