Chapter 15

Multiple Embryo Time-Lapse Imaging of Zebrafish Development

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Summary

Understanding the dynamics of developmental and cellular processes requires documentation of their changes with appropriate temporal and spatial resolution. Furthermore, simultaneous recording from a population of embryos under identical conditions allows statistical estimates of precision and variability to be made. This chapter describes a protocol for time-lapse microscopy of multiple embryos in parallel developing under tightly controlled conditions. This method is currently best suited to follow tissue-scale morphogenetic movements with temporal resolution in the minute range, for hours or even days. Applications of the method include the comparison of the dynamics of a process of interest between groups of wild-type embryos and their mutant siblings or between embryos treated with different chemical compounds. Temperature control allows for the investigation of the temperature dependence of a process of interest.

Key words: Zebrafish, Embryogenesis, Brightfield imaging, Time-lapse, Morphogenetic movements, Population statistics, Quantification, Temperature control.

1. Introduction

Time-lapse imaging of developing embryos has become a widely used tool in cell biological and developmental studies. Zebrafish embryos are well suited for this approach because their transparency and external development allow for easy visualization of a wide range of biological processes without adversely perturbing the embryo. As detailed elsewhere in this volume, techniques for observing single cells as well as cell populations in single embryos have reached a high level of sophistication. However, to enable the quantitative comparison of potentially noisy or variable processes

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within and between populations of embryos, multiple embryos need to be observed simultaneously. We here report a protocol for time-lapse imaging of multiple entire embryos throughout development. This setup is particularly useful for analysis of global morphogenetic processes on a population level.

For multiple embryo time-lapse imaging, embryos are held in defined positions in agarose molds and a motorized stage enables the recording of single images or *z*-stacks of individual embryos at defined time intervals. Temperature in the dish can be monitored throughout the entire experiment using a thermocouple device. Movies are generated from images of individual embryos, and used for further processing and analysis. The high number of embryos observed in one experiment allows statistical treatment of the data. The temporal resolution of the system, determined by the duration of an imaging cycle, is in the range of minutes, and embryos can be continually monitored for 30 h or longer **(Fig. 1)**. Brightfield imaging was the method of choice in most of our experiments. However, we anticipate that the power of this technique may increase considerably if combinations of different illumination settings are used, like multiple color fluorescence imaging, or combinations of brightfield and fluorescence imaging.

We have used this method to carry out an extensive characterization of the dynamics of zebrafish somitogenesis *(1)*, and we venture that this technique will be so versatile as to enable observation of a wide range of developmental processes. We anticipate

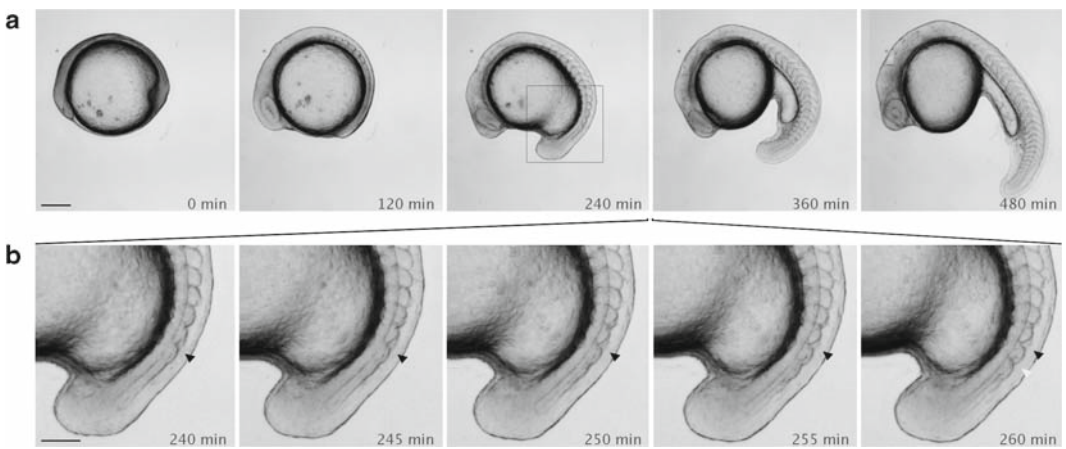

Fig. 1. Selected set of images from a time-lapse movie of a laterally mounted embryo. (**a**) Imaging of somitogenesis from bud stage until the 25-somite stage. The embryo shown is *nic*^{b107} homozygous *(2)*, and therefore genetically paralyzed, which makes it suited for imaging of late developmental stages over extended periods of time. *Scale bar* represents 200 μm. (**b**) Temporal resolution is in the range of minutes, which enables investigation of the dynamics of somite formation. A *black arrowhead* highlights the most recently formed somite boundary at 240 min. After approximately 20 min, a new somite boundary (*white arrowhead*) has formed. *Scalebar* represents 100 μm.

applications such as observation of the dynamics of eye morphogenesis, brain ventricle formation, epiboly, or other such general morphogenetic changes in early development.

2. Materials

2.1. Aluminum or Lucite Cones and Silicone Molds

1. Aluminum or Lucite plates (40 mm × 40 mm × 2 mm).
2. Drilling device from in-house workshop.
3. Silicone elastomer Sylgard 184 (Dow Corning).
4. Adhesive tape.
5. Medium-sized Petri dishes (55 mm × 15 mm).
6. 2% agarose in E3 medium.
7. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 10^{-5}% methylene blue (*3*).

2.2. Imaging

1. Manipulation tools for orienting embryos. We use eyelash hair on a pencil, blunted glass probes, slightly blunt watchmaker's forceps, or similar non-damaging probes.
2. Sharpened watchmaker's forceps (size 5) for removing chorions.
3. Large plastic Petri dishes (94 mm × 16 mm) coated with 2% agarose/E3 for dechorionation.
4. Microscope system with motorized stage. We use a Zeiss Axioskop 200M with a Zeiss MCU 28 motorized stage. Lenses, filter sets, phase rings and illumination settings depend on the desired imaging technique.
5. Digital camera. We use a Photometrics HQ Coolsnap Camera.
6. Software package for driving the motorized stage and for image acquisition. We use MetaMorph software (version 6.2r4).
7. Binocular dissection scope for manipulating and orienting embryos. We use an Olympus SZ40 with magnification ×0.67 – 4.0.

2.3. Temperature Control and Monitoring

1. Temperature control devices: air conditioning and heater. We heat the room with an electric radiator (AKO-ISMET, type T909 TSIII) and achieve temperature regulation with a built-in air conditioning system (Silent; Axair).
2. Device to ensure even mixing of air in imaging room. We use a rotary ventilator or fan.

3. Thermocouple. We use a K-type thermocouple (Voltcraft Plus K202).
4. Data logger and software for temperature recording (Voltcraft Plus K202, ThermoLogger).

2.4. Optional Materials for Specific Adaptations

1. 96-well deep well plate (nerbe plus) for further treatment of time-lapsed embryos.
2. Separating plastic plates for subdividing one Petri dish into up to four different chambers (53 mm × 10 mm × 2 mm), for observation of embryos treated with different chemicals.
3. Ethyl-*m*-aminobenzoate methanesulphonate (Tricaine; Sigma-Aldrich). Prepare as 4 mg/mL stock solution in 1% NaH_2PO_4 buffer, pH 7.4. Store at –20°C and avoid exposure to light. Anesthetic to assist observation of late developmental stages.
4. Heterozygous *nic*^{b107} carrier fish (*2*); an alternative to using anesthetic.
5. Danieau's buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.1–7.3 (*2*).

3. Methods

Before the start of the first time-lapse experiment, a number of devices that are not commercially available need to be prepared, such as silicone cones for production of agarose molds for embryo mounting (*see* **Subheading 3.1**) and separating plates for observation of differentially treated embryos (*see* **Subheading 3.4**, **step 2**). Furthermore, the microscope system (*see* **Subheading 3.2**) and temperature control devices, if required (*see* **Subheading 3.3**), need to be set up and checked for constant functioning before the start of imaging.

Because of the strong temperature dependence of most, if not all, developmental processes in the zebrafish embryo, care must be taken when comparing and interpreting imaging results from environments with unknown or variable temperatures. Nevertheless, the core imaging protocol can be run without temperature control (*see* **Subheading 3.2**). Controlling and recording the temperature also is covered below (*see* **Subheading 3.3**).

3.1. Production of Aluminum or Lucite Cones and Silicone Molds

1. Drill depressions into Lucite or aluminium plate. Size and shape of the depressions have to be adjusted to the desired developmental stage and orientation of the embryo during imaging (*see* **Note 1** and **Fig. 2 a**, **a′, b, b′**).

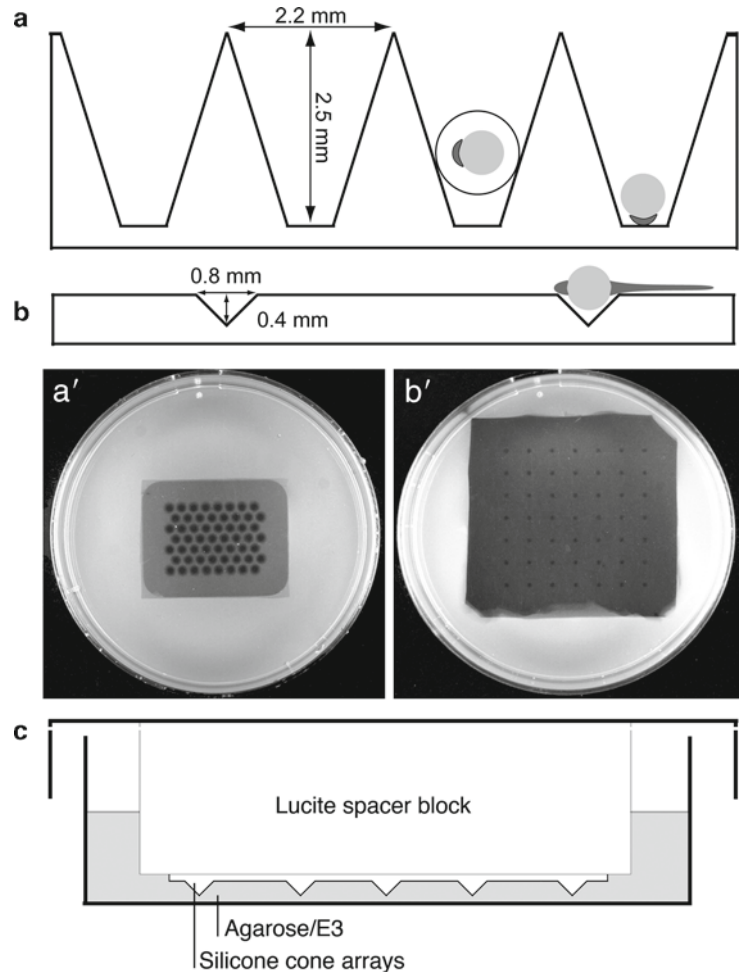

Fig. 2. Production of custom-milled agarose molds for imaging of zebrafish embryos. (**a**) Schematic drawing and dimensions of agarose molds suited for imaging embryos up to the 18-somite stage, as viewed from the side. (**a′**) Petri dish with ready-to-use array of agarose molds as depicted in (**a**), as viewed from above. (**b**) Schematic drawing and dimensions of agarose molds suited for imaging embryos from bud stage onward, as viewed from the side. (**b′**) Petri dish with ready-to-use array of agarose molds as depicted in (**b**), as viewed from above. (**c**) A silicone plate is fixed on a spacer block of Lucite glued into the lid of a Petri dish. Applying the lid to the agarose-containing Petri dish then creates molds of the desired size and spacing.

2. Mix the two components of the silicone elastomer kit with a spatula or similar device according to the manufacturer's instructions. Remove resulting air bubbles by centrifugation for 2 min at 860*g*.
3. Surround the depression plate with adhesive tape and cast a 5-mm layer of the silicone mixture onto the depression plate. Remove air bubbles by centrifugation for 1 min at 210*g* on plate buckets.

4. Let the silicone mixture solidify (2 h at 70°C or overnight at room temperature). The mixture will remain fluid for more than 12 h when stored at 4°C.
5. After the mixture has solidified, carefully remove the silicone plate with the cones from the depression plate. Remove excess silicone. The depression plate can be used repeatedly for production of silicone cone plates.

3.2. Imaging

1. Cast 6–8 mL of 2% agarose/E3 into a medium-sized Petri dish and place the silicone cone plate into the liquid agarose. Fixing of the silicone plate onto a spacer block of Lucite glued into the lid of the Petri dish is useful to minimize the thickness of the agarose layer between cone depressions and the bottom of the dish **Fig.** (**2c**).
2. Remove silicone cones after the agarose has set (*see* **Note 2**).
3. If required, dechorionate embryos with sharpened watchmaker's forceps in an agarose-coated Petri dish to avoid perturbation of the embryo resulting from adhesion to the plastic (*see* **Note 3** or **Subheading 3.6**).
4. Place one embryo in each conical well of the imaging dish when embryos have reached the desired developmental stage. Under a stereomicroscope set up right next to the time-lapse microscope, orient embryos as desired shortly before the beginning of recording (*see* **Note 4**), using slightly blunt forceps or some other gentle embryo manipulator.
5. Mount the Petri dish containing the oriented embryos onto the time-lapse microscope, and adjust illumination settings.
6. Use MetaMorph's MultiDimensional Acquisition tool to specify the variables of the time-lapse; these include number of time points, time interval between time points and, optionally, number and spacing of *z* planes (*see* **Notes 5** and **6**).
7. Select the positions to be monitored during the time-lapse using the manual control of the motorized stage, and save them via MetaMorph software.
8. Start the recording. It may be worthwhile to occasionally check the progress of the time-lapse.
9. Use the "Open Special/Build Stack" commands in MetaMorph to generate TIFF-stacks from the recorded images, and the "Make Movie" command to produce QuickTime movies (*see* **Note 7**).
10. The resulting stacks and movies as well as individual images are then used for further analysis, either by visual inspection or by automated image processing, depending on the experimental requirements.

3.3. Temperature Control

1. To obtain a stable temperature for the experiment, switch on heater and air conditioning system at least 2 h before starting the time-lapse. This allows benches, microscopes, and walls to reach equilibrium (*see* **Note 8**). Place empty imaging dish onto the microscope stage and switch on microscope, camera, and motorized stage (*see* **Note 9**).
2. Insert the temperature sensor of the thermocouple device into the agarose through a small hole in the lid after the dish containing the embryos has been mounted on the microscope (*see* **Note 10**).
3. Ensure air circulation around the body of the microscope (e.g., by placing a ventilator or fan in front of the microscope [*see* **Note 11**]).
4. Start temperature recording simultaneously with the time-lapse. Many commercially available thermocouples come with data logger software capable of monitoring temperature at specified intervals throughout the duration of the time-lapse. Save temperature data in a separate text file after the end of the time-lapse.

3.4. Further Treatment of Time-Lapsed Embryos

It may be important to identify the genotype or other distinguishing characteristic of the embryos for later correlation with the time-lapse movies.

1. Carefully remove the Petri dish from the stage after the time-lapse movies have been recorded.
2. Place embryos individually into a multi-well plate and let them develop until they have reached the desired stage.
3. Depending on the experimental requirements, these embryos can then be subject to higher magnification DIC brightfield or fluorescence microscopy. Alternatively, they can be fixed for immunostaining or *in situ* hybridization, or used for DNA extraction and subsequent polymerase chain reaction (PCR) genotyping *(3)*.

3.5. Differential Treatment with Chemicals in One Dish

If the experimental aim is the comparison of chemically treated and untreated embryos, two or four individual chambers can be created in one Petri dish with separating plates **(Fig. 3)**.

1. For creating four chambers, insert two plastic plates with small square incisions in the middle of one of the long sides **(Fig. 3a)**, which enables interdigitation of the two plates **(Fig. 3b)**.
2. For creating two chambers, grease three sides of a size-matched plastic plate with silicone grease, and position the plate in the Petri dish **(Fig. 3c)**.

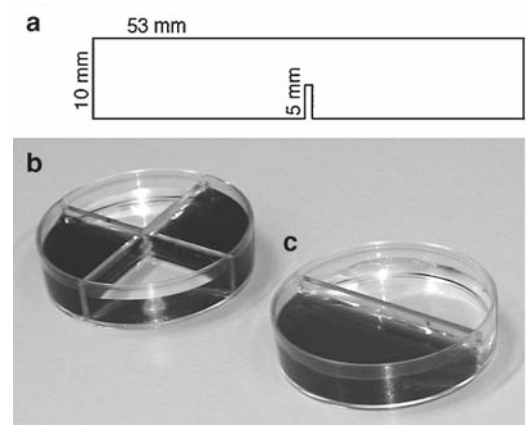

Fig. 3. Creation of multiple chambers in one Petri dish by insertion of separating plates. (**a**) Schematic drawing and dimensions of separating plastic plate with small square incision. (**b**) Four chambers are created by two interdigitating plastic plates. (**c**) Two chambers are created by one plastic plate. Different colored liquids may help to detect leaks between the chambers.

3. Check whether chambers are leak proof by filling differently colored liquids into the chambers and test for mixing after overnight incubation.
4. Use 4 mL of agarose/E3 for one of two chambers, and 2 mL for one of four chambers, and cover the agarose with E3.
5. Add the chemical to the desired experimental concentration. Calculate the amount of chemical to be added according to the total volume of agarose/E3 and E3.
6. Let the concentrations of chemicals in agarose/E3 and E3 equilibrate for at least 1 h before time-lapsing.

3.6. Documenting Late Developmental Stages

At approximately the 18-somite stage, zebrafish embryos start moving. At the same time, tail elongation results in bending of the axis as long as the embryos are in the chorion. Furthermore, shortly before the end of the segmentation process, embryos start to produce melanophores, which may preclude observation of some processes occurring beyond that stage (*4*). To accommodate these morphological changes in the imaging setup, integrate the following modifications into the core protocol (*see* **Subheadings 3.2** and **3.3**).

1. To accommodate the pronounced axial elongation following mid-somitogenesis stages, the distance between the silicone cones must be large enough (*see* **Note 12**). Use 0.5-mm deep conical depressions to fit the yolk of the embryo and guide tail elongation over the surface of the agarose (**Fig. 2b**).

2. The length of the zebrafish embryo at late-somitogenesis stages and beyond may be problematic if the entire embryo is to be documented. Choose a very low magnification objective, a digital camera with a large chip or an adapter to obtain a large enough field of view.
3. Cast agarose depression dishes as described above, but dissolve the agarose in 0.3X Danieau's buffer instead of E3. Cover the agarose with Danieau's and determine the volume of agarose and medium by weighing. Add Tricaine from the stock to a final concentration of approximately 0.016% (*see* **Note 13**) and let the anesthetic diffuse into the agarose for at least 30 min. Instead of chemical anesthetics, homozygous *nic*^{b107} embryos can be used, which are genetically paralyzed *(2)* (*see* **Note 14**).
4. Dechorionate embryos at any time before starting the recording using sharpened watchmaker forceps in an agarose-coated Petri dish. Transfer embryos to the depression dish and orient them laterally. If embryos are mounted at bud stage, the tail will elongate approximately in the direction of the tail bud. Orient all embryos with the bud pointing towards the same direction and set the region of imaging such that the tail will then elongate across the imaging area **(Fig. 1)**.

4. Notes

1. We found that depressions 2.2-mm wide and 2.5-mm deep **(Fig. 2a**, **a′)** are suited for imaging embryos both with and without chorion, either dorsally or laterally, respectively, up to the 18-somite stage *(1, 5)*. Conical depressions 0.8-mm wide and 0.4-mm deep **(Fig. 2b**, **b′)** are suited to image embryos laterally from bud stage onward. Finding the optimal depression design for other imaging purposes may require some experimentation. Number and arrangement of cones can be chosen as required.
2. For use on the same day, cover agarose molds with E3 medium immediately to prevent desiccation. A number of agarose molds can be prepared in advance and stored at 4°C for up to 2 wk.
3. Whether embryos need to be dechorionated or not depends on the scope of the experiment. Dechorionation may be advisable if embryos are to be mounted in an orientation that they do not normally adopt inside their chorions, such as dorsal imaging of somite stage embryos, which tend to lie on their sides inside the chorion. Furthermore, for all embryos to be imaged after the 16-somite stage, dechorionation is

necessary because tail outgrowth would otherwise result in bending of the embryo axis.

4. Allow sufficient time for dechorionation of embryos to start recording at the desired developmental stage. When mounting the embryos, take into account the morphogenetic changes that might alter the orientation of the embryo in the course of the time-lapse. Make sure the stereomicroscope and the time-lapse microscope are right next to each other to minimize transport distance between microscopes.
5. The number of images to be taken and saved in each round of imaging depends on the number of embryos and the number of *z* planes recorded, and this is the prime limiting factor of temporal resolution. In our setup, the time required for saving the individual images seemed to contribute the largest part to the time required to monitor one embryo. When imaging forty embryos and five *z* planes with 50 μm intervals per embryo, the minimal interval between time points is approximately 5 min. If the system cannot complete an imaging round in the time interval specified in the MetaMorph software, there will be no error message, but the system will go through repeated rounds of imaging without interruption. This results in disagreement of the supposed and real time interval, the latter being larger by an unknown amount. Therefore, it is important to match the time resolution of the experiment to the other parameters determining the number of images per round before the beginning of recording. Higher temporal resolution may be achieved by increasing the speed of the motorized stage, by reducing the number of embryos and the number of *z* planes per stack and by reducing file size of individual images.
6. The speed of the motorized stage must be adjusted to avoid dislodging the embryos form their oriented positions. We found a speed of approximately 4 mm/s appropriate for the cones described here.
7. Manual generation of stacks or movies in MetaMorph can be time-consuming if a large number of embryos is to be analyzed, and if selection of different *z* planes has to be performed. To automate this procedure, we used the freely available ImageJ software to produce focused and time-stamped stacks and movies *(1)*. The ImageJ Stack Focuser plugin (http://rsb.info.nih.gov/ij/plugins/stack-focuser.html) selects and combines the in-focus areas from different *z* planes of one time point. All focused images from one position are combined into a time-stack, time-stamped and saved both as a TIFF-stack and QuickTime movie. We wrote a multithreaded Java plugin, combining Stack Focuser, Time Stamper, and Movie Writer plugins to simultaneously

process data from multiple embryos in parallel. This plugin has been tested and optimized for brightfield images. If a similar method is to be applied to other image types, modifications in the plugin may be required.

8. This method works very well for a small room with low traffic of personnel, and as long as there is monitoring of temperature, the experiments are well controlled. If these requirements cannot be met, temperature control in the sample dish could also be achieved with a heated microscope stage, or similar device. Make sure the stereomicroscope and the time-lapse microscope are temperature-equilibrated to avoid accidental hot or cold starts.
9. The heat emitted by these devices contributes significantly to the temperature in the imaging dish.
10. This should be performed before the positions to be imaged are selected, as insertion of the probe after position selection may slightly displace the dish. It is important to ensure unhindered movement of the temperature probe when the motorized stage is moving, in order to avoid dragging of the imaging dish by the probe.
11. This prevents heat convection from camera and/or microscope to the sample and is necessary to stabilize temperature and avoid excessive heating and consequent desiccation of the imaging dish.
12. We found that a distance of 4 mm between the cones is enough to film embryos until the end of somitogenesis.
13. If titrated to the lowest concentration that inhibits embryo movement, we found that Tricaine has only minor influence on the dynamics of development in imaging experiments that lasted more than 15 h. However, higher doses of this anesthetic applied for long periods can significantly slow down development, and we recommend titrating the lab stock of Tricaine before any experimental series by carefully comparing the staging at selected time-points with untreated littermates.
14. These fish lack functional nicotinic acetylcholine receptors and are therefore non-motile. However, they otherwise develop fairly normally for the first days and we did not find significant differences in the dynamics of somitogenesis compared with wild-type fish. Nevertheless, the usefulness of this fish line will have to be evaluated separately for every experiment. In addition, when a number of genetic mutants are to be assayed, crossing multiple mutations into the nic^{b107} background can become a significant task and using Tricaine to immobilize embryos may be a more straightforward approach under these circumstances.

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