Introduction

Laboratory 1: Stages of Zebrafish Development

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Developmental biology is a broad field of study that encompasses embryology (the generation and ultimate placement of zygote-derived cells in an organism) as well as all other subsequent changes that take place after birth.[1] In the current laboratory session, embryonic stages of development in zebrafish (*Danio rerio*) are examined.

During embryonic development, zebrafish undergo meroblastic cleavage. This type of cleavage pattern requires intracellular calcium and typically gives rise to two microscopically distinct regions: a multi-cellular region that will eventually become the organism and a yolk region (Figure 1).[1-3] The yolk acts as a source of nourishment for the organism as the multi-cellular region continues to proliferate. As development progresses, anatomical structures including eyes, ears, notochord, and somites become visible (Figure 2).[3] Embryonic development in zebrafish lends itself well to observation, an assessment based purely on the transparent nature of the embryo. Because the embryo is transparent, all internal structures can easily be seen as development proceeds.

Although zebrafish embryos have distinct internal structures, the size is still quite small (approximately 0.7 mm) and requires use of a microscope.[4] In this lab, there are two types of applicable microscopes, the first of which is the dissecting microscope (DM) (Figure 3A). As the name implies, this microscope is useful for *microscopic* dissections. In zebrafish, for example, a DM could be used for dechorionation, the process of removing the outer most protective layer of the embryo.[5] This type of dissection is possible due to the two lens arrangement within the microscope which allows for three-dimensional viewing of the embryo.[6]

The second type of microscope is the compound microscope (CM) (Figure 3B). CMs are more applicable for two-dimensional viewing of samples, as in histological tissue sections.[6] CMs also differ from DMs in their total magnification. Whereas DMs will allow for visualization of general organ structure, CMs allow visualization of intracellular components such as nuclei.[6] In each microscope, however, adjustment of both objective and lens within the eye piece will permit some range of zoom function.

Zebrafish are valuable organisms because their development is similar to other vertebrate animals. This similarity is advantageous because it allows for extrapolation of experimental data in zebrafish to human embryos. In zebrafish experiments, the embryo characteristics most frequently exploited by scientists are *permeability* and the *transparent nature* of the outer most layer. With regard to permeability, the embryo allows free passage of many small molecules including ethanol, retinoic acid, and other pharmaceuticals.[1] In studies by Rocke et al[7] and Barros et al[8], for example, cardiac and neurologic development were examined in the presence of pharmacologic agents capable of entering the embryo. Studies such as these try to identify adverse effects that both new and common drugs may elicit in developing embryos.

With regard to transparency, studies are aimed at monitoring cellular processes in *real-time*. In a study by Kim et al[9], for instance, structural changes to mitochondria during apoptosis (programmed cell death) were visualized using green fluorescent protein (GFP) markers. The fluorescent color was easily seen through the transparent embryo.

With the information above as evidence, it is clear that zebrafish provide a unique way to study developmental biology. Although much is known about the organism, it is likely that zebrafish will continue to expand upon current concepts and inspire research.

Figure 1. Early Stage of Meroblastic Cleavage.

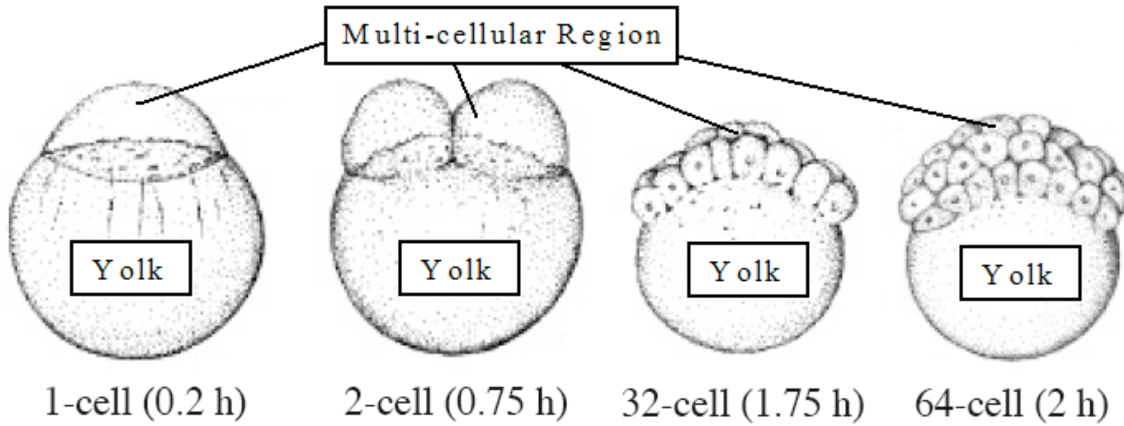


Figure 2. Formation of Anatomical Structures in Zebrafish Embryos.

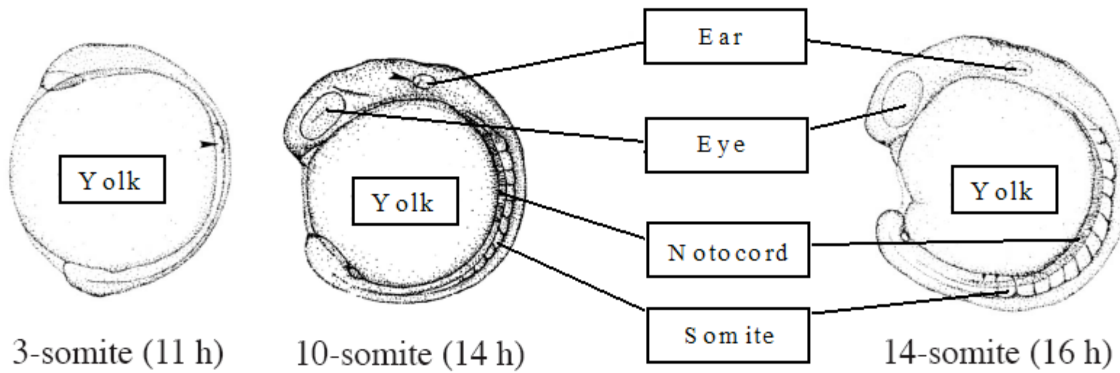
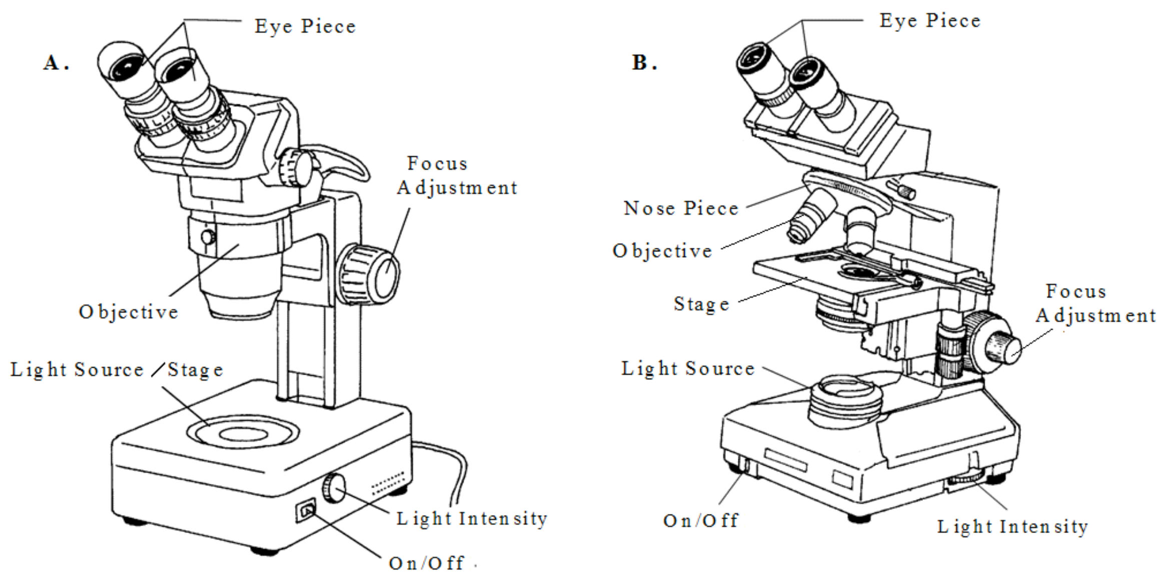


Figure 3. Microscopes. A. Dissecting Microscope, B. Compound Microscope.



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Protocol for Laboratory 1: Stages of zebrafish development

The goal of this laboratory is to learn the stages of zebrafish development, and to use dissecting and compound microscopes.

1. Tools

You will use several tools for the experiment today:

Dissecting stereomicroscope and a compound microscope. We will go over how to use these before we start. If you have not used one of these before and need some help, please let me know and I will give you an introduction. We will be using these for almost every experiment this semester

A pair of fine forceps. Each student will have their own pair to use throughout the semester. They are quite fragile and expensive, so please take care of them. Use them only when working with the embryos. These are fairly dull, and you may prefer to sharpen them with a stone. You can use these to dechorionate (take the shell off) the embryos and to push them around if you are careful.

Embryo loops. These are for orienting and pushing the embryos around. These are made with fishing line, capillary tubes, and super glue. You should make several of these for yourself.

Slides. Live embryos must be mounted on slides in methyl cellulose before you can view them on the compound microscope. We have two kinds of glass depression slides that you can use. See the attached protocol for how to mount the embryos.

2. Staging zebrafish embryos

You have been given a petri dish with four-six stages of embryos. Your challenge is to separate embryos at different stages of development into different dishes, and to determine their stage.

Dissecting microscope

First look at them under the stereo dissecting microscope (low power). Put the petri dish on the stage, and turn on the light underneath the stage. Adjust the mirror so you get even illumination across the field of vision. This is called brightfield microscopy.

As you look at the embryos, think about the following issues, and write about them in your laboratory report.

Use the attached staging sheets to stage your embryos. Note characteristic features of each stage that help you identify it.

What are the limitations of an observational approach to developmental biology? What are some of its advantages? Think about developmental structures and processes that are easy and hard to observe.

Play with the setting of the light and the mirror on your microscope. Are their settings that make it easier or harder to see specific structures? For example, try turning the mirror so light is not going directly through the embryo. This is pseudo dark field microscopy. Is it easier or harder to see the somites? Can you count how many somites your embryos have?

An important skill for the course will be the ability to work with the embryo underneath the dissecting microscope. There are several things you can do even with the simple tools you have today. Most of the stages you are looking at still have their chorion-can you take it off (dechorionate the embryo) with your fine forceps? Does dechorionating make it easier of harder to see structures within the fish? When do you first see movement of the embryo? What kinds of movements can you see? When does it start to respond to touch?

Compound microscope

Next take one of your embryos and look at it under different lenses of the compound microscope. You can use one that is dechorionated, or one that is still in the chorion (but choose one with a clean chorion). Follow the directions for mounting the embryos using methyl cellulose.

Adjust the compound microscope for bright field microscopy (see microscopy intro), and observe the embryos at different magnifications using the different objectives on the compound microscope. How does what you see differ from what you could see using the dissecting microscope?

Now adjust the microscope for dark field microscopy. In this method, Put a coin on top of the light source-a big coin for a low power objective, and small coin for a high power objective. This will create a ring of light that is illuminating your embryo-this is called dark field microscopy. How does this change the brightness and contrast of the image you see? Are their structures that are easier to see in dark field versus bright field, and vice versa?

Special techniques for light microscopy

I want you to understand these techniques in enough detail that you can evaluate the disadvantages and advantages of using them in imaging zebrafish embryos. You should also be able to use each of them, and know the function of each of the major components in the light path. For example, if you sit down at a microscope and someone has changed the settings, you should be able to make the adjustments to get the kind of light microscopy you need.

This is my overview, which is meant to fill in some gaps in the background reading. If you want to learn this in even more detail-there is a great website. Visit http://micro.magnet.fsu.edu/primer/index.html

1. Brightfield
This is the most direct and probably most familiar method of light microscopy. In this method, the light comes directly through the sample. To adjust your stereomicroscope for bright field, you should turn on the light, and then adjust the mirror in the base until you see an equal, bright level of light throughout the field of view. For the compound microscope, you should adjust the microscope for Koehler illumination (see handout or vade mecum CD). You can see the light path for a bright field microscope in Figure 6 of your microscope guide.

2. Darkfield
This method can be used to better image specimens that are transparent and unstained because it generates contrast. Its disadvantages are that it can create glare, and it does not generate an accurate geometrical image of the sample because the light is distorted.

In darkfield microscopy the image of the specimen is formed completely by light scattered by the specimen. Therefore, the specimen will look bright, while the background will look dark. To do this, one must block all of the light that would come directly through or around the specimen. All of the light that reaches the specimen will then be coming at very high angles (meaning from the sides), and the only light that reaches the objective will be the light that is scattered because it hit the fish.

For the stereomicroscope, you can create a dark field image by turning off the source of illumination, and using the room light that is coming in from the sides. In some stereomicroscopes, you can also do this by moving the mirror out of position.

For the compound microscope, you can place a coin on top of the light source. Choose a coin that leaves only a narrow ring of light around the image you see through the eyepiece. You will need a different size coin for each objective.

On the research compound microscope in my laboratory, you can turn the condenser to “D” for darkfield.

3. Phase Contrast
When light is diffracted by passing through a specimen, its phase is slightly altered. Phase contrast microscopy converts this change in phase, which we cannot see, into changes in amplitude, which we can see. This results in higher contrast.

The conversion from phase differences to amplitude differences is accomplished by speeding up the light that has not been affected by the sample so that it is ~1/2 wavelength out of phase with the light that was diffracted by the sample. This results in interference between the unaffected and diffracted light, which in turn causes differences in amplitude. For example, in one kind of phase contrast, there will negative interference in places where the sample is dense, and these areas will appear darker than the background. Areas that are thin, or have a low refractive index will appear lighter than the background.

In phase contrast, a condenser annulus is placed between the light source and the sample, creating a cone of light illuminating the sample. A phase plate placed after the objective is used to shift the wavelength and reduce the amplitude of the undeviated light passing through the sample. These two additions to the light microscope cause interference between the undeviated light and diffracted light and changes in amplitude that we can see.

Unfortunately, the teaching stereomicroscopes and compound scopes are not equipped for phase contrast. We will have to go on a field trip to my lab.

On my research compound scope, the condenser should be set to #1 for phase contrast, this puts the condenser annulus in place. The turret should be set to any number between 4-8-this are open positions. The phase plate is incorporated into the objectives.

4. Differential interference contrast (DIC) microscopy

DIC is similar to phase contrast in that both methods translate differences in phase into differences in amplitude, increasing the contrast of translucent samples. However, they use different methods to do this. While phase contrast compares light that is diffracted by the sample to light that is not diffracted by the sample, DIC compares light that passes through two closely spaced points in the sample. In other words, DIC translates differences in refractive index or density between two nearby points on the sample into changes in amplitude. For example, if a tissue in zebrafish is denser than the neighboring tissue, it will appear dark, whereas if it is less dense it will appear light.

DIC has a quite complicated light path. I will just hit the highlights here.

Light first passes through a polarizer, which polarizes the light. Now all the light traveling to the sample is oriented in the same direction. Next, the light passes through a Nomarski or Wollaston prism, which splits a beam of light into two orthogonal beams of light with slightly different paths. The beams pass through the sample approximately very close to each other-closer than the resolving power of the objective. Because they are not vibrating in the same plane, they do

not interfere with each other. Any differences between the tissue the two beams pass through, such as differences in refractive index, thickness, slope, will cause differences between the light paths of the two beams. A second Wollaston prism, located in the objective, then recombines the two beams. Finally, a second polarizing filter, called the analyzer, puts the two beams of light back into the same orientation. If the beams have traveled different optical paths, they will now interfere with each other. Typically, one side of a detail will appear dark because the gradient between the two beams of light is from a more dense area to a less dense area, and reciprocally, the other side of the detail will appear light. There may also be differences in color. The resulting image looks three dimensional, but the shading affect is really due to optical differences rather than the topology of the sample.

The teaching microscopes are not equipped for DIC, so we will use this method on our field trip. For DIC, the turret should be set at 1; this puts the analyzer in place. The condenser should be set at II.

Anesthetizing zebrafish embryos and larva

Once the zebrafish start moving (at ~17 somite stage) it may help to anaesthetize them before observing them under the microscope

Chemical safety: Do not splash the anesthetic around. It contains a nasty chemical, 3-aminobenzoic acid ethyl ester (MS-222), which is categorized as an irritant. Be careful, wear gloves and wipe up any spills promptly and completely. If the anesthetic solution comes in contact with your skin or eyes, rinse with copious amounts of water, and tell the instructor.

Protocol:

1. You may want to dechorionate the embryos you want to observe. This is not absolutely necessary as long as what you want to see is not obscured by the chorion. Once the embryo is dechorionated, it is much more fragile, so be gentle.

2. Use a pipet to transfer one or a few embryos or larvae to a dish containing a solution of MS-222 or Tricaine. Anesthesia will occur in less than a minute. If you are using a stereomicroscope, you can observe the fish directly in the petri dish. Continue to steps 5 and 6. If you are using a compound microscope, continue to step 3.

3. Transfer the animals in a droplet of the drug to a glass depression slide containing a small amount of methyl cellulose (see mounting protocol). Continue as described in the methyl cellulose mounting protocol.

4. During you observations, make sure to keep the zebrafish covered with solution. The lights of the microscope can cause rapid evaporation.

5. Do not keep the embryos under anaesthesia for more than 30 minutes, or they may not be able to recover.

6. When finished, transfer the embryos back into fish water to allow them to recover.

Koehler illumination

Setting your compound microscope up for Koehler illumination will give you the best possible resolution. These steps are taken directly from the Vade Mecum CD that comes with your book.

1. Place the lowest power objective into place. You can do this by manually turning the nose ring of the microscope. You should always start out with the lowest power objective. It helps you find your sample more easily, and will avoid accidents. For example, some thick slides may not fit under higher-powered objectives, and the objective or the slide might get broken.

2. Put your slide into place and use the focus knob to bring the sample into focus.

3. Close down the field diaphragm.

4. Focus the condenser by moving the condenser up and down while looking through the eye pieces. Adjust until the edges of the field diaphragm are in focus.

5. Open the field diaphragm until light just fills the field of view.

6. Adjust the iris diaphragm.

Most effective way-you should try this at least once. Take out one of the eye pieces (oculars) of the microscope. You are now looking down at the back of the objective. Adjust the iris diaphragm until 2/3 of the back lens of the objective is filled with light.

Alternately, you can adjust the iris diaphragm by less exact methods. One way is to just open and close it until you find the setting where you judge that your sample has the best resolution. Another was is to close the iris diaphragm just until the light you see through the eyepiece starts to dim.

How does opening and closing the iris diaphragm affect the resolution and contrast of the image you see? Could you use this to your advantage?

Mounting zebrafish embryos using methylcellulose

Methyl cellulose is a convenient way to mount live embryos for observation under the compound microscope. It is very gentle and non-toxic, and the embryos can easily be recovered.

Chemical safety: No hazards are listed for methyl cellulose, and no protective gear is suggested, but you may want to wear gloves. If it comes in contact with your skin or eyes, wash affected area with soap and copious amounts of water and inform instructor.

Protocol:

1. Using an embryo loop, place a small dab of 3% methylcellulose in the center of the depression of a glass depression slide. Don't just drop the methyl cellulose on, push it down, otherwise it will float away.

2. Dechorionate and/or anaethetize the embryos as necessary. If you are planning to photograph the embryos, you should dechorionate all but cleavage stages, and anaesthetize embryos at any stage where there is movement.

3. Place the embryo on top of the methylcellulose, and layer fish water on top to fill the depression.

4. Move the slide under the dissecting microscope and gently push the embryo into the methylcellulose with an embryo loop until it is secure. Use the loop to position the embryo in the orientation that you want. Now you are ready to look under the compound microscope.

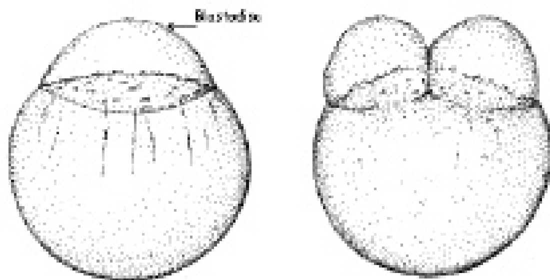
5. Be careful to keep the embryo covered with water during your observations.

6. To recover the embryos, move the slide back to a dissecting microscope, and use a pipet to transfer the embryos back to a petri dish containing fish water.

Developmental Biology Laboratory
Observations of the Zebrafish Embryo

Adapted from http://www.neuro.uoregon.edu/k12/zfk12.html

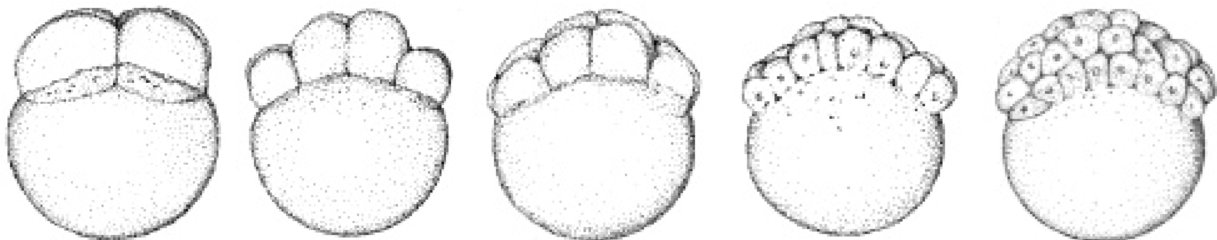
Zygote Period (0-.75h)



1-cell (0.2 h) 2-cell (0.75 h)

Cytoplasm streams toward animal pole to form the blastodisc.

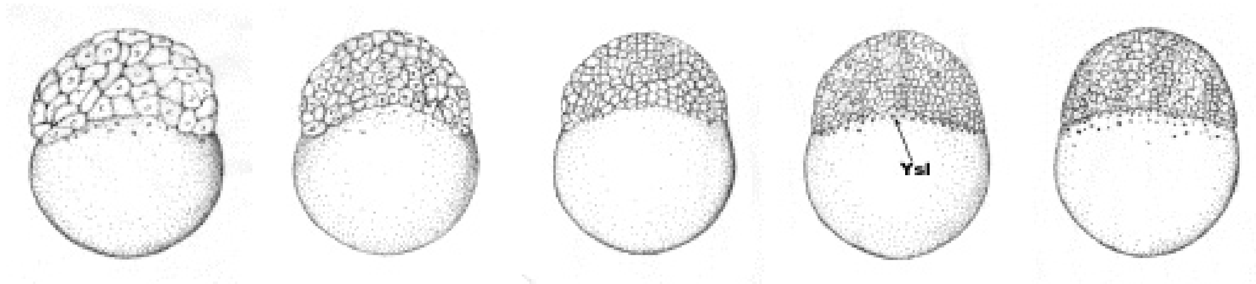
Cleavage Period (0.75-2.2 h)



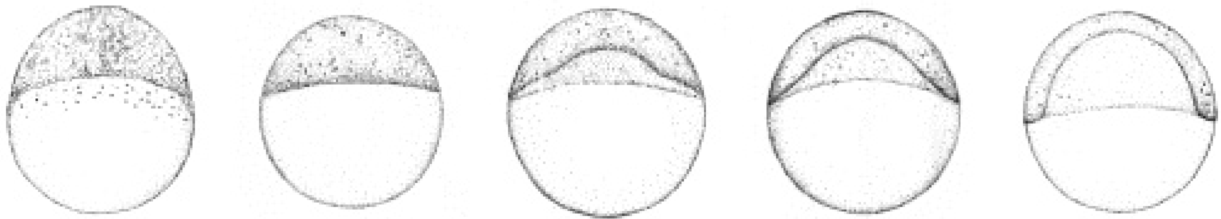
4-cell (1 h) 8-cell (1.25 h) 16-cell (1.5 h) 32-cell (1.75 h) 64-cell (2 h)

During this period, the first 6 cleavages occur. The cells, or blastomeres, divide synchronously at about 15 minute intervals.

Blastula Period (2.25-5.25 h)



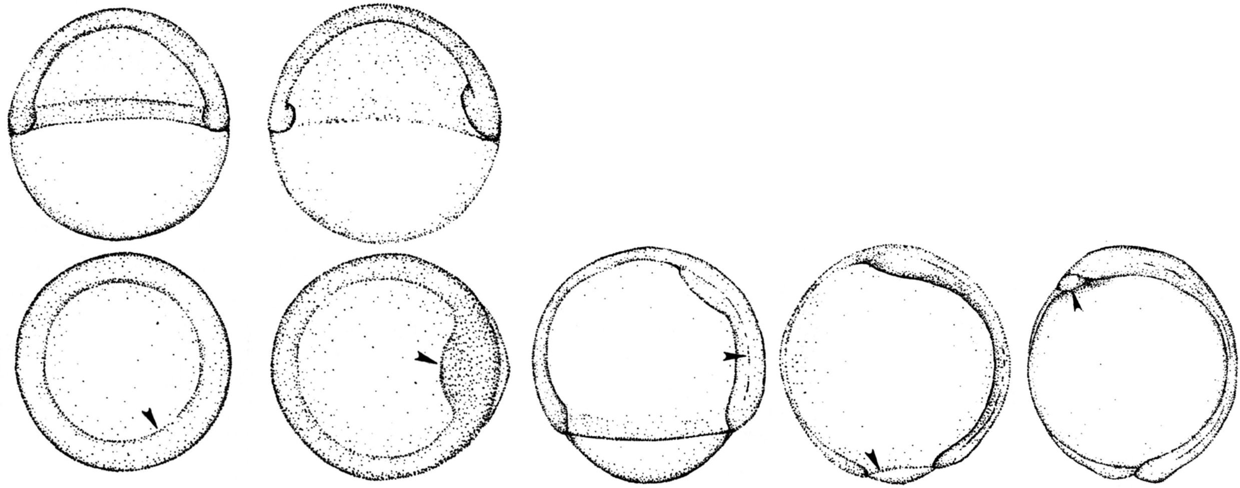
128-cell(2.25 h) 256-cell (2.5 h) 512-cell (2.75 h) 1k-cell (3 h) high (3.3 h)



oblong (3.7 h) sphere (4 h) dome (4.3 h) 30%-epiboly(4.7h) (50% epiboly (5.3h)

Midblastula transition occurs at the 10th cleavage. At this division, cell membranes do not form between cells of the bottom, marginal, row of blastomeres, and thereafter, it develops into the “yolk syncytial layer (YSL)” of the yolk cell. After midblastula, transition cell divisions are asynchronous. Margin reaches 30% epiboly.

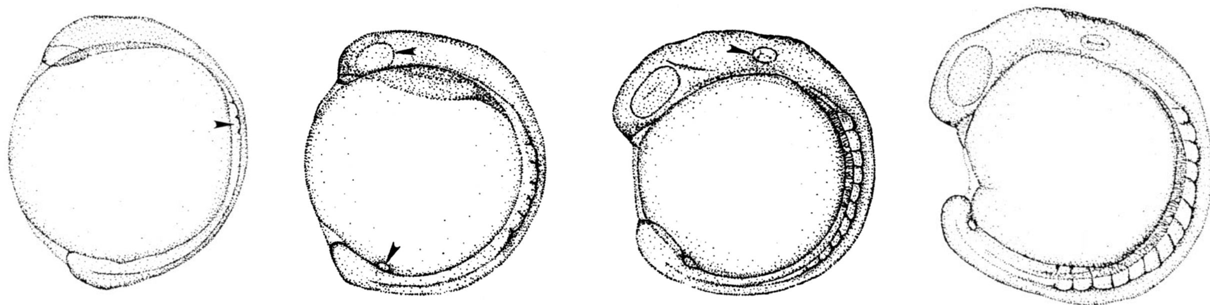
Gastrula Period (5.3-10 h)



germ ring (5.7 h) shield (6 h) 75% epiboly (8 h) 90% epiboly (9 h) bud (10 h)

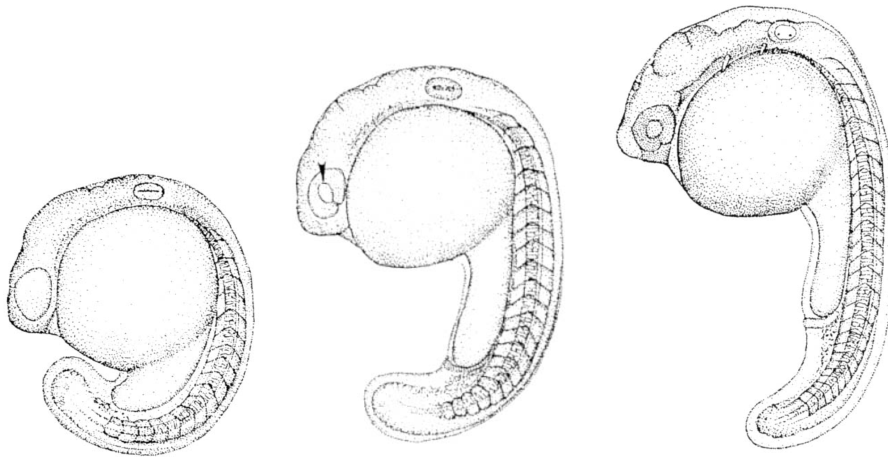
Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis through the end of epiboly.

Segmentation Period (10-24 h)



3-somite (11 h) 6-somite (12 h) 10-somite (14 h) 14-somite (16 h)

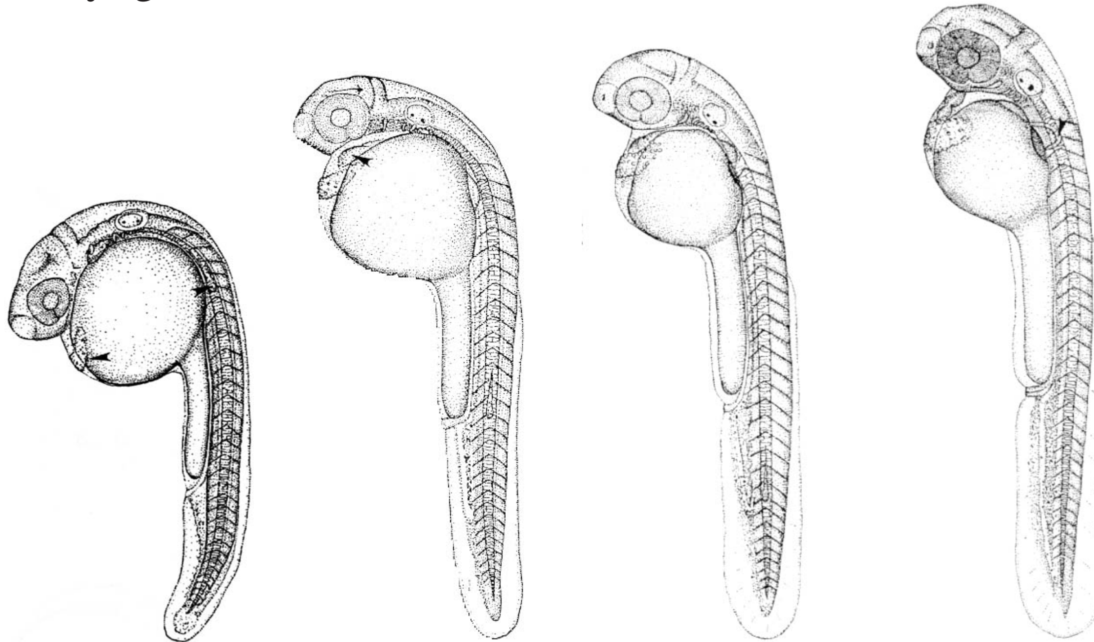
Segmentation Period (continued)



18-somite (18 h)   21-somite (19.5 h)   26-somite (22 h)

Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears

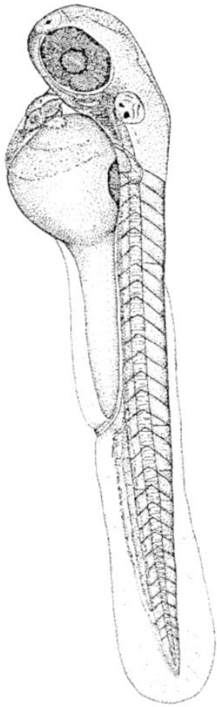
Pharyngula Period (24-48 h)

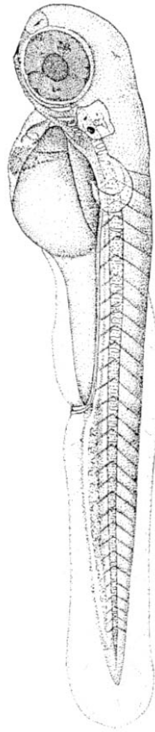


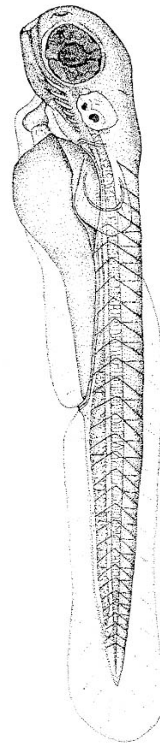
Prim-6 (25 h)   Prim-16 (31 h)   Prim-22 (35 h)   High-pec (42 h)

Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and fins begin development.

Hatching Period (48-72 h)







long-pec (48 h) pec-fin (60 h) protruding mouth (72 h)

Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral fin; hatching occurs asynchronously. At 72 h, swim bladder inflates; food-seeking and active avoidance behaviors.

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The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*

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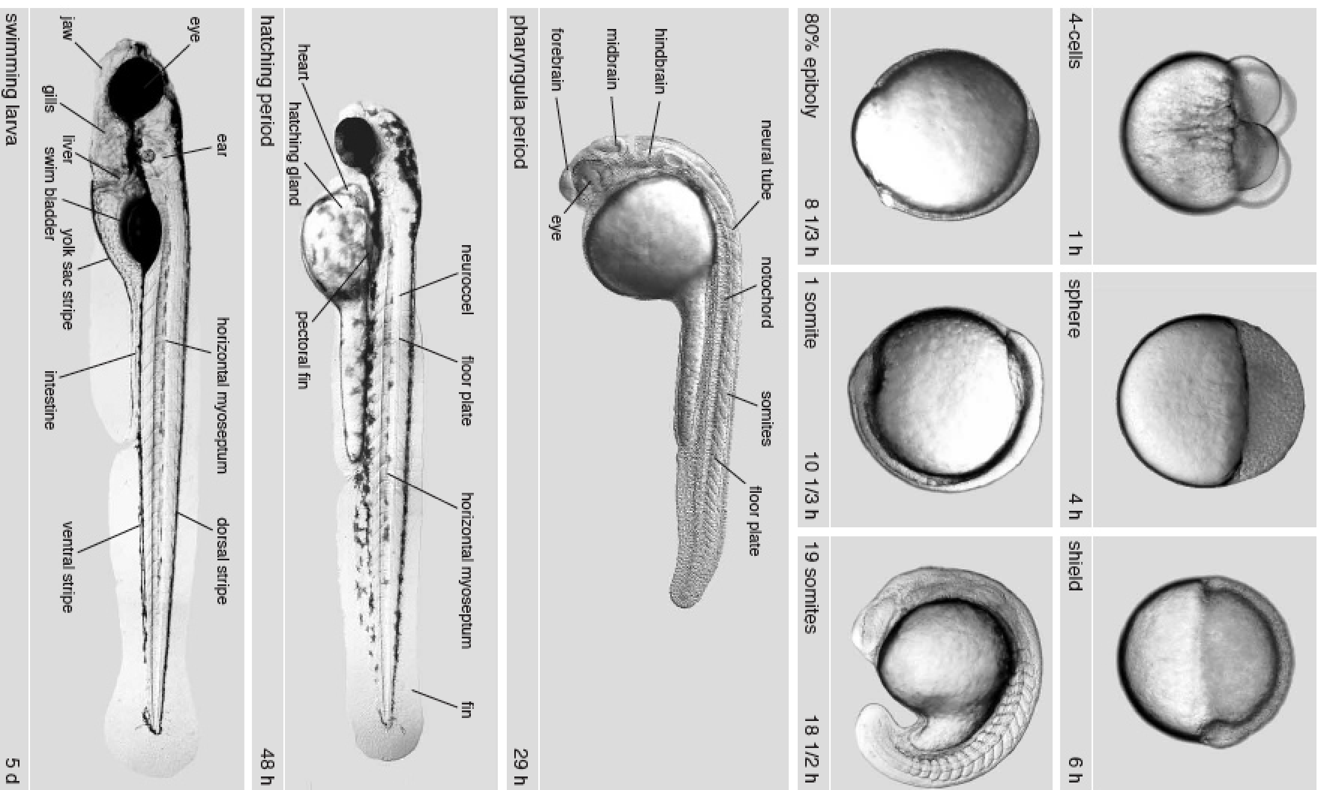

Fig. 1. Living embryos of relevant stages during the first 24 hours of development and of the approximate age during the three screening periods. The structures that were on the checklist are marked.