

Introduction to the zebrafish

Materials for Sunday and Monday – to help introduce the zebrafish, stages of embryonic developmental and mutants. Chuck Kimmel

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- I. Sunday** evening introductory lecture. “Status of the zebrafish for vertebrate genetics and development”, Accompanying notes including a **homework** problem for you to solve.
- II. Monday** morning lab introduction. “Watching embryos develop – a primer for the first lab”. Accompanying notes.
- III. Monday Lab** notes “Exploring the zebrafish embryo: stages and mutants”,
- IV.** “About the Quicktime Movies”. Find the movies – showing early development – on the Mac in the lab for you to review on your own, and/or to copy to a CD.

Figures: A set of 3 figures from the Metscher and Ahlberg paper, and a set of 5 drawings of embryos and larvae made by Cosima Fabian, when she was a student in Tübingen, to serve as guides for the first large-scale ENU screen with zebrafish. All but the last one was published in the P. Haffter et al. (1996) paper in the issue of *Development* describing the screens. Look through the entire issue to see the scope of the endeavor. Find this citation, and other good stuff on **ZFIN**; <http://zfish.uoregon.edu/>. Cosima’s drawings were also reproduced in Appendix 2 of the book “Zebrafish”, Eds. C. Nüsslein-Volhard & R. Dahm, Oxford University Press (2002).

We separately provide copies for you of the 1995 Kimmel et al. paper “Stages of embryonic development of the zebrafish”, which will be useful in the lab. A PDF version is on the lab’s Mac and also PDF files of the following two introductory papers:

- B.D. Metscher & P.E. Ahlberg** (1999) Zebrafish in context: Uses of a laboratory model in comparative studies. *Dev. Biol.* **210**, 1-14.
- J. Langeland & C.B. Kimmel** (1997), Chapter 19: Fishes, in “Embryology: Constructing the Organism” eds. S.F. Gilbert & A.M. Raunio, eds., 537 pp.

I. Sunday evening

Status of the zebrafish as a model for vertebrate genetics and development

Some features of the zebrafish and its embryo make it an attractive system for learning about development and how genes control development.

1. The zebrafish as a genetic model:

George Streisinger: Founder of the 'modern era' of studies:
} External fertilization. Clutch size. Generation time.

- } Construction of adults homozygous at every locus in the genome, and clones (hence isogenic, with uniform genetic background) (1981).
- } With Charline Walker and others: First methods for mutagenesis and genetic analysis, including mapping (1983). Now we have a completely resolved genetic map (John Postlethwait et al., 1995).
- } Using gynogenesis by “Early Pressure” (EP), procedures for F1 screens making mutant hunts very efficient.

Homework problem (Do it! & prepare for Charline’s Tuesday morning lesson on EP): George, Charline and others worked out the “EP procedure” of gynogenesis that makes many mutations homozygous in the EP progeny of heterozygous females. Using EP shows that the *golden* locus is located far away from its centromere (among the EP progeny from a heterozygous *gol⁻/gol⁺* mom, only 5% are homozygous mutant, *gol⁻/gol⁻*) and that zebrafish have high chiasma interference (meaning one crossover event during meiosis suppresses subsequent crossovers along the same chromosome in the same cell; Streisinger et al., 1986). At first, not knowing where the *gol* gene maps or about chiasma interference, George was fooled into thinking meiosis occurs differently in zebrafish than in other creatures: Namely, he mistakenly thought that sister chromatids separated during the first meiotic division and homologues separated during the second division. Of course, the opposite sequence is actually what happens. **Explain** how George could have made such an error (chromosome drawings will help), and propose a way to correct it. [An answer will come tomorrow]

2. Mutants! useful tools to understand how development works, how the nervous system is wired, and many other problems.

- } First developmental mutants in zebrafish identified by such screens. Charline finds *spadetail*, *cyclops*.
- } Janni Nusslein-Volhard & Wolfgang Driever: The large scale genetic screens.
- } Now many mutations, identifying hundreds of developmentally acting genes are available, and are being characterized and mapped. The list includes over 500 viral insertional mutants, this method permitting very rapid cloning of the mutated locus.

3. Genomics

- } A rich genetic map that includes hundreds of cloned genes and mutations, thousands of coding sequences, ESTs, and microsattelites. Efficient mapping strategies.
- } Other genomics infrastructure, making it straightforward to characterize genes and their functions, and to molecularly identify mutated genes --- hundreds of thousands of EST sequences, about a thousand pictures of RNA in situ hybridization patterns, and a nearly completely sequenced genome.
- } Discovery and significance of a whole genome duplication, not shared with tetrapods, occurring in the early lineage of zebrafish and other teleosts.

- } Is the zebrafish ‘tetraploid’? In fact, evolution after the duplication includes gene loss and gene subfunctionalization. The *cyclops/squint* example, from Talbot, of subfunctionalization of duplicated genes.

4. Where does the zebrafish fit in, considering vertebrate evolution?

- } Sordino et al.: The pattern of *Hox* gene nested expression in the zebrafish paired fin bud is different from that in the tetrapod limb bud, but like that proposed for the tetrapod ancestor. Is zebrafish a good model organism for a tetrapod ancestry?
- } An incorrect notion about evolutionary intermediates – a linear evolutionary pathway from one model organism to another: *C. elegans* -> fruit fly -> zebrafish -> frog -> chicken -> mouse -> human.
- } Rather, the zebrafish is a twig on the teleost branch (>20k teleost species!) of the vertebrate evolutionary tree (review: Metscher & Ahlberg, 1999 (PDF available); see the figures reproduced from this paper.)

5. The zebrafish as a model for vertebrate development:

- } Optical clarity, facilitating phenotypic analyses such as lineage analyses and time lapse analysis of morphogenesis, easily manipulable (e.g in transplantation studies). Embryogenesis is rapid.
- } Tissues develop that first have relatively small numbers of cells. e.g. the nervous system, including the segmentally organized hindbrain.
- } Fate mapping establishes where these tissues come from in the early embryo, and that the general topology of the fate map is highly conserved among chordates.
- } High conservation of developmental regulatory genes, their expression patterns, and in many instances, their functions as well.

6. An example of a large scale {‘global’} analysis of developmental pattern.

- } Morphogenesis of the notochord, an example where we analyze the behaviors of a whole field of developing dorsal mesodermal cells from confocal time lapse recordings. The data indicate that a particular cellular interaction called ‘mediolateral intercalation behavior’ proposed by R. Keller, quantitatively accounts for the entire morphogenetic behavior of the deep mesoderm during notochord convergence (narrowing) and extension (elongation). Applying the method to the *no tail* mutant, in which convergence is disrupted, produced a surprising result (from Glickman et al., 2003).

II. I. Monday morning

Watching embryos develop – a primer for the first lab

In the Stages paper (see ZFIN for better images), embryonic development is divided into a set of developmental **periods** (e.g. the period of gastrulation), and further subdivided into an open-ended series of **stages**. We use time-lapse movies in this class meeting (see also “About the Quicktime Movies”, below) to introduce what is happening during the early developmental periods:

1-cell (or zygote period) (0-0.7 hr)

- } Newly fertilized egg. The non-yolky blastomere segregates towards the animal pole. You can watch the cytoplasmic segregation, the formation of the first blastomere and then its division.

Cleavage (1-2.2 h)

- } Rapid cell divisions, without cell growth, occur within the blastodisc. Cell number tells you the stage.
- } Meroblastic cleavage. Stereotyped but not invariant division patterns. Lineage tracing. Metasynchronous timing. Deep cells and the enveloping layer (EVL).

Blastula (2.2-5.2 h)

- } Cell size and shape of the blastodisc tells you the stage.
- } Yolk syncytial layer (YSL) formation in the midblastula, and the likely function of the dorsal YSL as a Nieuwkoop organizing center. Midblastula transition and the origin of zygotic gene expression. Early ‘random’ (?) cell movement.
- } Morphogenesis begins in the late blastula. Epiboly by radial intercalation -- scatter of clonally-related cells. The blastoderm margin as zone of lower cell-scattering.

Gastrulation (5.2-10h)

- } Epiboly continues and conveniently indicates stage.
- } Beneath the EVL the blastoderm begins marginal ingression to develop two layers. The outer epiblast feeds the inner hypoblast (or mesendoderm).
- } Both layers undergo convergence and extension. The first organ, the notochord, becomes delineated by establishment of the axial/paraxial boundaries in the mesoderm (eventually the notochord/somite boundary).

III. Monday

In the Lab: Exploring the embryo – stages and mutants

WHAT WE DO: We do three ‘experiments’ (sets of observations, really) to answer the three questions below. Our purpose is to begin to become familiar with the early developmental pattern, normal and abnormal, of our zebrafish *Danio rerio*.

EXPERIMENT ONE

Question: How would you characterize the division pattern of the early cleavages?

With a large bore pipette transfer a few 2- or 4-cell embryos, within their chorions, into a small petri dish, cover them entirely with water, and observe them with the dissecting microscope. Use transmitted, not reflected, illumination. Place the illuminating lamp rather close to the microscope, so as to keep these tropical fish embryos warm as they develop. Getting the proper temperature with just the light as a warmer is tricky. An ideal temperature is 28.5°C, several degrees above ambient room temperature. At this temperature the early cleavages are at 15 min intervals, and the rate is slower if the incubation temperature is lower. Abnormal cleavages will occur if the embryo is much too cold.

You will be able to gently roll the individual embryos about in the dish using a small probe, but take care not to damage the chorion. Using this technique you can observe the cleaving cells from different angles, which will help you figure out the cell arrangements and the way the cleavages occur. You will easily see that the early cleavages are "**meroblastic**" (see the Glossary in the back of the Staging Series paper for the definition of this and other specialized terms). Carry out a short **cell lineage analysis**: Follow what is happening during several successive divisions, and if you are a wizard you can try to keep track of more than a single embryo as you do this. Notice that divisions often seem to occur along stereotyped "cleavage planes". Take notes. Make sketches, but work quickly for things may happen fast while you are not watching. Record the times of your observations. You may find that the first 5 or so cleavages occur in a regular, but not an invariant, pattern. Can you come up with a simple rule that predicts cleavage planes? What is the nature of variability among individuals?

Stop during late cleavage, after about the 6th cell division - as the cells will become so small and numerous that you will find it increasingly difficult to keep track of the cleavage pattern. At this time we could follow cells for a few more divisions using DIC (Nomarski) optics on the compound microscope available. With DIC how could you tell, other than by counting cells, whether the midblastula transition has happened or not?

EXPERIMENT TWO:

Question: How do we use morphology to determine developmental stage?

Dechorionate manually (if possible – the older the embryos are, the easier it is). Anesthetize (if the movements of the embryo make this necessary). Use the staging series to determine the stages of available embryos as exactly as you can at various times during the day. Come back at night to catch the lovely segmentation period embryos that you would otherwise miss. How synchronous is development within a clutch? What different characteristic tissues and cell types

can you see at each stage using just the dissecting microscope? using DIC on the compound microscope?

EXPERIMENT THREE:

Question: What are the phenotypes of 'mystery mutant' embryos?

By the pharyngula period the primary body organs have formed and many cell types have begun differentiation. For example, you will notice that pharyngula embryos can move. The movement requires both functional nerve cells and muscle cells. Hence this time is an important one for screening mutagenized fish for patterning mutations. Prove this to your own satisfaction by identifying and examining in detail what has gone wrong with pharyngula mystery mutant embryos. We'll supply beakers containing embryos of each strain that were obtained by intercrossing two fish heterozygous for a single mutation. If the mutations are recessive and fully penetrant what fraction of the embryos does Mendel tell us to expect will look mutant?

You'll need to carefully compare mutants with their phenotypically wild type siblings, making your observations with the dissecting microscope, the tool with which the mutations were initially discovered. Key features of the wild type pharyngula ('24 h') are included in the set of Fabian drawings.

Roll the embryos around, within their chorions at first and inspect from every angle. Head on. Tail on. Side views. Dechorionate. **Discover!** After the class has had a reasonable time to make and record observation we will demonstrate the phenotypes using DIC, and discuss. Don't miss the opportunity to make discovery on your own before this happens!

IV. About the Quicktime Movies

1. The low magnification overview of development is provided by the “**Zebrafish Flipbook**” (R. Karlstrom & D. Kane, 1996) covering cleavage through segmentation stages. Does the movie provide evidence for **i.** a loss of synchronization of cleavage divisions in the blastula? **ii.** an inwards movement of cells at or near the blastoderm margin during gastrulation/epiboly? **iii.** the dogma that vertebrate segmentation occurs in an anterior-posterior wave?

2. A set of Nomarski movies (ysl, midblastula transition, marginal ingression, and deep shield, shallow shield) are collected in the “Nomarski” folder. I made these movies for the Woods Hole course, using a Zeiss axiophot and Cytos software to capture images at series of z-levels. I then assembled single plane movies at interesting levels into the Quicktime movie format.

ysl: High magnification (40x objective) focus on the marginal blastoderm cells to illustrate yolk syncytial layer (YSL) formation beginning at the 512-cell stage and continuing through late blastula. The blastoderm is to the bottom and the yolk cell to the top. The interval between frames is 30 sec. The specialized YSL nuclear divisions unaccompanied by cytokinesis are evident: nuclei disappear as mitosis begins, and later, pairs of daughter nuclei reform in a common cytoplasm. The first disappearance you see is the tenth cleavage (i.e. completing the 10th zygotic cell cycle). Note that YSL nuclear divisions are synchronous, and cycling is more rapid than for blastomeres also present in the field (see the Kimmel & Law paper on YSL formation, 1983, also Kane et. al., 1992).

midblastula transition: 20x objective, 20 seconds between frames. Focus is on the dividing cells of the blastula, beginning at the 9th cleavage division, the last fairly synchronous one, that produces a 512-cell blastoderm. Thereafter you can see that cycle times slow down and any semblance of synchrony is gradually lost. Note also that during MBT the cells gradually acquire motility -- you can see cytoplasmic blebbing during interphases, and pseudopodia forming late in the movie, but not before MBT, at the beginning of the movie. (see the Kane & Kimmel paper, 1993).

marginal ingression: 20x objective, 30 seconds between frames, yolk cell to the top. The YSL is evident during the whole course of the movie, which begins in the late blastula and continues into the early gastrula period. Blastoderm coherent streaming in the vegetal direction (towards the top of the field) is evident throughout the whole course of the movie; this is the movement we term epiboly. The view is of the lateral blastoderm, the dorsal side is out of the field to the left; later in the movie you can detect some convergence movement of cells towards the left (towards dorsal. Part way through the movie you see a slight global contraction (the beginning of gastrulation?), and then (but not earlier) ranks of the marginal blastomeres (i.e. the leading blastomeres) continue to disappear from this level as they internalize.

shallow shield, deep shield: 40x objective, 30 seconds between frames. These two movies, of the dorsal margin beginning in the early gastrula, were made from a multifocus recording of a single embryo, exactly the same time frames are used in both movies, with the "deep" movie showing a level of focus 18 μm deep to the "shallow" movie, i.e. a little more than a single cell

diameter at this stage. (The shield is more than 3 cell diameters thick at these times). Near the end of the movies the field is repositioned without a change in focus, so you can follow the margin, advancing by epiboly, for a longer time. It is possible for you to relocate the same cells after this upwards jump. In the shallow movie you can see the group of forerunner cells (see Melby et al., 1995; Cooper and deAmico, 1996) that lead the advance of blastoderm epiboly, and are a convenient marker of the dorsal midline. The forerunner cells show very active protrusive activity, as do many blastoderm cells just at the margin. Many cell divisions are visible as well. The cellular movements of the blastoderm at the dorsal midline have not yet been well described in the literature. John Shih, when he was a postdoc in Scott Fraser's lab, characterized an "ingression" movement of shield cells located near the margin (unpublished, or so I think). By ingression we mean that cells intercalate as individuals to deeper blastoderm levels, whereas involution means a more coherent flow of cells at the margin. As you can see in these two films, the movements are certainly more complicated than you observe in the "marginal ingression" movie, which is of the lateral blastoderm, rather than the dorsal aspect where the shield is present. Note that in the "shallow" movie, to either side of the midline you can see cells disappearing at the margin, as typical of involution. Do you detect the same movement just at the midline margin in the center of the field? What about at corresponding positions in the "deep" movie? In the "deep" movie, at the midline particularly, you can trace many individual single cells through the frames that recede from the margin as it advances due epiboly. (That is, as the margin advances rapidly downwards, individual cells at this focal plane become further and further translocated way from the margin. Where do you suppose the cells replacing them are coming from? Do you see the same behavior in the same relative position in the "shallow" movie?)

3. H2aGFP shallow, deep, and division, make up a set of three movies made from a single 4d recording during epiboly and early gastrulation, using confocal imaging of cells expressing GFP driven by the histone H2a promotor. Only nuclei are visible. The transgenic cell strain was made in the lab of Jose Campos-Ortega and is available from ZIRC. The movies were made by Martha Jones in the Kimmel lab. **H2aGFP Shallow** is a shallow optical plane of section, it includes the EVL present peripherally in the field (large nuclei), surrounding the deep cell epiblast layer to the center of the field (smaller nuclei). Note that all cell movement, EVL and deep epiblast, is downward, roughly toward the vegetal pole and away from the animal pole. The deep epiblast cells are outrunning the EVL – as explained by the fact they are feeding cells into the hypoblast at the margin as well as spreading the epiblast vegetally. **H2aGFP Deep** includes the hypoblast (mesoderm/endoderm-forming) in the center of the field. You can see that behavior is quite different from the surrounding epiblast. Cells move up, away from the margin and toward the animal pole. Note, at the margin off center, how the epiblast seems to feed into the hypoblast by marginal ingression. Following individual cells (nuclei) would require the original 4d recording itself, so you can move up and down the layers. **H2aGFP Division** is a zoom-in on a region of epiblast from the shallow movie letting you nicely see mitoses. See how the nuclei about to divide are large, as compared with others nearby. Suppose this movie is not right on dorsal, but is off to the left or right. Watching the epiblast drift, where do you suppose the dorsal midline is located in the embryo – to the left or right of the field of view?

4. The "Nathalia & Richard" folder collects a set of state-of-the art animations showing convergence and extension in the dorsal mesoderm during gastrulation and early segmentation. Nathalia Glickman made the original time lapse multiplane recordings with a confocal microscope

for her Ph. D. thesis work at the University of Oregon (Glickman et al., 2003). She used Mark Cooper's method of cell labeling with Bodipy ceramide (which shows up the intercellular spaces, leaving the cell interiors dark). In all of the movies the interval between frames is 90 sec. Richard Adams (Cambridge University, UK) developed the software to facilitate analysis of the huge amount of data these records contain, and to display the results in as movies. Play through the set in the following order:

cells: this is a single depth from the original multiplane confocal recording. Play it through to the end and examine the late stages first, so you can see the tissues that are forming. The hypoblast (mesendoderm layer) is in focus in the center of the field, and is surrounded by epiblast – the boundary between the two layers shows up as a circular white haze around the edge of the field. You can easily recognize the notochord rudiment in the center of the field, 2-3 cells wide at this stage. The notochord is flanked by a distinctive row of adaxial cells, that will go on to develop slow muscle. Two somite furrows are also visible in the paraxial mesoderm that forms the bulk of the body wall muscle (fast muscle). Play through the movie to see how all of these structures form.

tracks: This movie was made by tracking cells frame by frame from the same original movie. Each track shows the pathway of movement of a single cell. The length of the tracks show speed: how far the cell has moved during a twenty min period.

stripe: A horizontal stripe of cells is selected out so you can easily contrast convergence and extension movements in the notochord vs. the paraxial mesoderm. Which seems to extend the most? Which seems to converge the most?

sparks: Here the blue flashes show where cell neighbor changes, due intercalations, are occurring within the notochord domain in another WT embryo. According to Ray Keller, convergence and extension in the dorsal mesoderm is driven by two processes, together called mediolateral intercalation behavior (MIB): 1. Polarized protrusive activity – cells protrude processes in a polarized fashion, along the mediolateral (ML) axis that make contact with new neighboring cells located along this axis. 2. Balanced tension – by contracting these same processes the cells exert tension upon one another, and the field contracts along the ML axis (convergence). Old neighbors are driven apart and, particularly in the notochord domain, the neighbor losses occur primarily along the anterior-posterior (AP) axis, elongating this axis (extension).

WT and *ntl* painted stripes: In these two animations the cells are painted different colors according to their initial ML positions in the field, making a set of vertical (AP) rows. In the WT, as the field undergoes convergence and extension you can see that these rows do not intermix very much. However in the *no tail* mutant, the balanced tension part of MIB doesn't seem to work. The cells extensively intermix and convergence is missing until late in the sequence. Interestingly, in spite of loss of convergence, the mutant cell field still extends at least as well as in WT; this behavior is certainly not predicted by the Keller MIB hypothesis.