“All the Zebrafish News That’s Fit to Print”

THE ZEBRAFISH SCIENCE MONITOR

Volume 2 Issue 2 | Monday, March 1, 1993 | Eugene, Oregon, USA

IMPROVED METHODS FOR *IN VIVO* DETECTION OF TRANSIENT EXPRESSION IN ZEBRAFISH EGGS MICROINJECTED WITH FIREFLY LUCIFERASE GENE

By Tibor Papp, Ferenc Muller and Ferenc Erdelyi; Institute for Molecular Genetics, Agricultural Biotechnology Center, Godollo, Hungary

The use of the firefly luciferase gene as an *in vivo* reporter in fish was first reported by Gibbs *et al.* (1991) and Alestrom *et al.* (1991). Our procedures are modified versions of the latter method.

We used as a vector the plasmid, pCMVl (a gift from Pat Gibbs, Washington State University, Pullman), which contains the firefly luciferase gene (de Wet *et al.*, 1987) driven by the human cytomegalovirus IE1 promoter (Boshart *et al.*, 1985). The measurements were made with a Betamatic V (Kontron) scintillation counter.

Protocol #1. Plasmid Coinjected with Substrate

1. Collect zebrafish eggs from aquaria into small, plastic Petri dishes (20-30 eggs/dish) containing Holtfreter solution.
2. While holding eggs with a “mouth-pipette”, microinject them by introducing 1.5 nl solution containing 1 g/ l plasmid and 0.25 mM luciferin (Sigma) under a stereo microscope (according to our experience there is no need to use expensive micromanipulators, injecting could be performed faster and easier by hand).
3. Incubate the eggs at RT until they reach early blastula stage (eggs are prone to mechanical damage before this stage).
4. While the eggs are developing set your scintillation counter according to the following protocol:

Setting	Value
Preset time:	0.1 min.
Low count reject:	No
Data mode:	cpm
Windows:	H3
Window optimization:	No
SCR survey:	C/A
Luminescence:	SURVEY
Sample replicate:	MULTIPLE
Sample repeat:	3
Sample delay::	No

5. Transfer embryos into scintillation cuvettes containing 5 ml of Holtfreter solution or dechlorinated tap-water. (Non-dechorionated eggs can be pooled for the first screening, however dechorionated eggs should always be incubated individually.)
6. According to our experience controls show luminescence readings below 30. All positive readings should be above 30, but values in the range of thousand are not rare (some counters give specific error messages beyond a certain level of luminescence).
7. Let embryos develop in the cuvettes, by changing their solution

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CHARACTERIZATION OF A CELL LINE DERIVED FROM ZEBRAFISH (*BRACHYDANIO RERIO*) EMBRYOS

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During the last decade, zebrafish (*Brachydanio rerio*) have emerged as a novel and attractive system to study embryogenesis and organogenesis in vertebrates. The main reason is that both extensive genetic studies and detailed embryological analysis are possible using this small tropical fresh water teleost. However, in vitro analysis using cell culture or molecular genetics are still far less advanced than in other vertebrate systems.

We report the generation and characterization of a fibroblast like cell line, ZF4, derived from one day old zebrafish embryos. The hyperploid cell line has been stable in multiple passages for more than two years now and is the first zebrafish cell line that can be maintained in conventional medium containing mammalian serum.

Using a series of plasmids for expression of a marker gene, we evaluate in ZF4 cells the relative strength of expression from several different viral, fish and mammalian promoters. Stable integration can be obtained by using G418 selection. We hope that our cell line will be a useful tool for the analysis of gene regulation in zebrafish.

THE ZEBRAFISH SCIENCE MONITOR

MONTE WESTERFIELD, Editor
PAT EDWARDS, Publications Coordinator

...an informal vehicle dedicated to communicating zebrafish news. References to information appearing in the Monitor should be made as personal communications and only if explicit permission of the authors is obtained.

Published at the Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403 USA

From the Editor

The long awaited new edition of *The Zebrafish Book* is finally ready! About time, huh?

It contains expanded versions of chapters from the first edition and a new chapter on cellular methods, including microinjection, transplantation and ablation of single cells.

Like the first edition, the new book is printed in a loose leaf format to make it easy to update. We intend the book to be a living document that grows and improves with time. However, this will require input from you. Please address corrections and additions to us.

An order form is included with this issue of the *Monitor*. A special note for labs outside the US: we can get a reduced postage rate for large orders. So, we encourage you to order all the books you will need for your lab as a unit, rather than placing several individual orders.

Happy fishing!

LETTERS TO THE EDITOR

To the Editor:

I write to argue that the *Zebrafish Science Monitor* should continue to remain invisible in the "official" published scientific literature. As I recall, our initial guideline was that the *Monitor* was not to be cited. But here are some happenings:

Recently I persuaded to a colleague to remove a *Monitor* citation from a draft of a review article. The citation was innocuous, we both agreed, but I argued that including it would set a precedent that could turn out to be dangerous to the community and the primary role that the *Monitor* plays in facilitating communication within it.

I then heard indirectly that another colleague was reluctant to put an abstract of interesting work into the *Monitor*, not because he wanted to hide the work, but because he was worried that a top journal would not then be interested in publishing it later, since the work was already announced in a publication. This is just what the no citation policy is meant to head off.

Lastly, a paper that I anonymously reviewed cited among the regular references, an innocuous technical contribution in the *Monitor* from their own lab. I suggested in my review that this was not an appropriate citation. If we stick to the no citation policy then some techniques already described in the *Monitor* will have to be described again in the formal literature. This seems unavoidable.

I hope that in a "sharing and caring" spirit, the use of *the Monitor* to abstract new unpublished work of scientific as well as technical interest will continue to increase. The no citation policy is crucially important to help this happen. It might be appropriate if the *Monitor* included in each issue a reminder notice of the policy.

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THE ZEBRAFISH MIDBLASTULA TRANSITION

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The zebrafish midblastula transition (MBT) begins at cycle-10. It is characterized by cell cycle lengthening, loss of cell synchrony, activation transcription, and appearance of cell motility. Superseding a 15 min oscillator that controls the first nine cycles, the nucleocytoplasmic ratio appears to govern the MBT. This timing mechanism operates cell-autonomously: Clones of labeled cells initiate cell cycle lengthening independently of neighbors but dependent on immediate lineal ancestors. Unequal divisions, when they occur, produce asymmetric cell cycle lengthening based the volume of each daughter. During several cycles after the MBT begins, cycle length is correlated with the reciprocal of the blastomere volume, suggesting a continuation of cell cycle regulation by the nucleocytoplasmic ratio during an interval we term the 'MBT period'. (In preparation).

FISH R US

Developmental biology experiments at home have brought baby boys to Bill Trevarrow and Jeremy Wegner.

Attila Adam, Ferenc Erdelyi, Ferenc Muller Tibor Papp and László Orbán keep the fish jumping at the Institute of Molecular Genetics in Godollo, Hungary.

Robert Ho will take zebrafish to Princeton when he starts his new lab next summer.

Improved Methods... *(cont'd from Page 1)*

every day. Use dechlorinated tap-water after hatching. (There are considerably more deformed embryos among the positives, than among the rest. For measurements on the 2nd day or later see Protocol II.)

Protocol #2. Eggs Injected with Plasmid Only and Incubated in Substrate

Expression gradually decreases during the time of development partly due to loss of plasmids and due to depletion of substrate. The latter problem can be solved by incubation of eggs in luciferin solution. Luciferin penetrates easily into the embryos and larvae without additional reagents and doesn't seem to harm the embryos.

1-4. Follow the steps detailed in Protocol I, except omit luciferin from the solution used for microinjection.

5. Transfer embryos into 0.75 ml Eppendorf tubes (short type) containing 100 l of 0.5 mM luciferin solution and group three tubes into a scintillation vial. (For decreasing the cost of detection, ATP, AcCoA, MgSO4 and glycylglycine can be neglected without substantial decrease in activity.)

6. Measure luminescence (50-60 cuvettes/hour in our counter), select positive groups. Separate Eppendorf tubes from vials showing expression into new ones and measure again. (Earlier we have tried to pool embryos in vials without using tubes and to transfer them by wide bore pipettes. The survival rate increased considerably after switching to the tube method.)

7. Transfer embryos into hatching tanks and test remaining luciferin solution for luminescence caused by lack of embryo residues. Pool filtrate through a 0.2 m Millipore filter and store in freezer until next use. (Luciferin solutions can be safely reused twice, except when contaminated by deterioration products causing turbidity).

In our experience the first positives appeared at about four hours after fertilization and expression could be detected even at 72 hours. We have successfully used the procedure described by Gibbs (1991) to confirm the data obtained with scintillation counters.

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RNA EXTRACTION FROM ZEBRAFISH EMBRYOS

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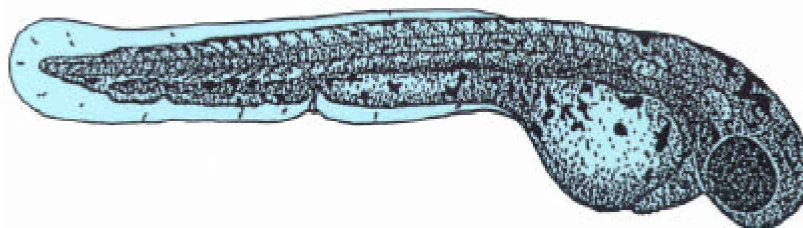
We have recently modified a LiCl RNA extraction protocol (Auffray and Rougeon, *Eur. J. Biochem.* **107**:303-314, 1980), to suite our needs in RNA extraction from ZF embryos. With the protocol described below we have routinely obtained approximately 10 micrograms/ 60 ZF embryos. The procedure is simple and convenient, as all steps are carried out in microfuge tubes.

1. Collect about 60 ZF embryos in sterile microfuge tube and remove as much liquid as possible.
2. Add 0.8 ml Sol. I (3 M LiCl, 6 M UREA, 0.1% SDS, 10 mM NaAc pH 5.5, 200 U/ml Heparin) and homogenize the embryos using a syringe and a 21 g needle 10 times. Keep on ice for at least 16 hrs (in the cold room).
3. Spin in microfuge for 15 min. at 4 C, resuspend pellet in 1 ml Sol. II (4 M LiCl, 8 M Urea) and spin again. Repeat step 3.
4. Resuspend pellet in 0.2 ml Sol. III (0.1 M NaAc pH 5.5, 0.1% SDS), extract twice with an equal volume of phenol:chloroform (1:1), and once with chloroform. Precipitate with 70% ethanol and 0.3 M NaAc.
5. Resuspend the RNA pellet in water and determine the OD. We have used this RNA directly for Northern analysis.



ZEBRAFISH CODON USAGE TABLE

By Anat Yarden; Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel.

The pattern of choices between synonymous codons varies from one gene to another according to the type of genome the gene occurs in (NAR 9, r43, 1981). Thus, codon usage may be biased in a species specific manner. Therefor, the codon usage table of an organism may prove to be useful for designing degenerate oligonucleotide primers for PCR, cloning, etc. In addition, knowledge of codon usage may yield a wide range of information about an open reading frame, such as whether an open reading frame is indeed likely to be a gene (*J. Heredity* **83**:239, 1992).

Today codon usage tables are available for many species through anonymous FTP site (132.183.190.10) from Dr. Michael Cherry (CHERRY@FRODO.MGH.HARVARD.EDU) (see also *NAR 20 Suppl*., 2111-2118, 1992). However, a zebrafish codon usage table has not yet been assembled.

I have pulled out of the genebank the available zebrafish sequences (GenBank release 74.0, EMBL release 33.0), and sorted the sequence of exonic fragments from them. Those exonic fragments were used to generate a zebrafish codon usage table by using the "codonfrequency" program available through GCG.

Table 1 lists the genes which were included in this analysis (coding sequences from additional gene fragments were also used, but are not listed). The zebrafish codon usage table shown in Table 2 includes the total number of times a specific codon appeared in all the fragments analyzed (appears in brackets) and the preferred ratio by which the various codons were used for a specific amino acid. Additional sequences, when available, can be added periodically to the table in order to increase its statistical value.

Table 1. List of Genes Used for the Zebrafish Codon Usage Analysis

Gene	Description
msh-D	muscle segment homeobox
zf-cad1	caudal homeobox
epen	ependymin
krox-20	zinc finger protein (krox-20)
antp	antennapedia class zf'26'
pax(zf-a)	*drosophila* paired homolog
alpha-tropo	skeletal muscle alpha-tropomyosin
hox(zf-22)	homeobox hox2.2
hox(zf-61)	homeobox hox(zf-61)
hox(zf-114)	homeobox hox (zf-114)
krox-24	zinc finger protein (krox-24)
hox(zf-21)	homeobox hox(zf-21)
zf-54	homeotic zf-54
eng	engrailed-like homeobox
zn-cad	N-cadherin (Bitzur, Kam & Geiger, unpublished)

Table 2. Zebrafish Codon Usage Table

Amino acid	Codon	Ratio (count)	Amino acid	Codon	Ratio (count)
Gly	GGG	0.18 (41)	Trp	TGG	1.00 (51)
	GGA	0.31 (70)	Stop	TGA	0.41 (9)
	GGT	0.21 (49)		TAG	0.23 (5)
	GGC	0.30 (68)		TAA	0.36 (8)
Glu	GAG	0.71 (212)	Cys	TGT	0.47 (32)
	GAA	0.29 (86)		TGC	0.53 (36)
Asp	GAT	0.39 (75)	Tyr	TAT	0.38 (54)
	GAC	0.61 (115)		TAC	0.63 (90)
Val	GTG	0.39 (67)	Leu	TTG	0.13 (43)
	GTA	0.15 (26)		TTA	0.09 (30)
	GTT	0.20 (35)	Phe	TTT	0.39 (59)
	GTC	0.26 (46)		TTC	0.61 (91)
Ala	GCG	0.16 (41)	Ser	TCG	0.08 (30)
	GCA	0.22 (56)		TCA	0.20 (76)
	GCT	0.31 (78)		TCT	0.18 (70)
	GCC	0.31 (79)		TCC	0.20 (78)
Arg	AGG	0.15 (49)	Arg	CGG	0.14 (43)
	AGA	0.24 (76)		CGA	0.14 (45)
Ser	AGT	0.15 (57)		CGT	0.11 (35)
	AGC	0.20 (76)		CGC	0.22 (70)
Lys	AAG	0.47 (120)	Gln	CAG	0.64 (125)
	AAA	0.53 (137)		CAA	0.36 (70)
Asn	AAT	0.36 (73)	His	CAT	0.39 (44)
	AAC	0.64 (132)		CAC	0.61 (69)
Met	ATG	1.00 (101)	Leu	CTG	0.34 (111)
Ile	ATA	0.23 (41)		CTA	0.08 (28)
	ATT	0.28 (50)		CTT	0.15 (49)
	ATC	0.49 (86)		CTC	0.21 (70)
Thr	ACG	0.15 (42)	Pro	CCG	0.16 (39)
	ACA	0.26 (71)		CCA	0.27 (66)
	ACT	0.23 (62)		CCT	0.24 (59)

RNA *IN SITU* HYBRIDIZATION PROCEDURE FOR WHOLE ZEBRAFISH EMBRYOS

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(Herrmann, B.G. (1991) *Development* **113**:913-917)

Many protocols for RNA *in situ* hybridization of whole vertebrate embryos have been published recently (Harland, *Methods in Cell Biology*, **36**:685-695, 1991; Herrmann, *Development* **113**:913-917, 1991; Schulte-Merker et al., *Zebrafish Science Monitor* **2**:8-9, 1992). Most of these protocols require a sophisticated set of treatments and extensive washing steps resulting in poor tissue preservation.

We report here a simple, but sensitive protocol for Zebrafish embryos which avoids treatments with RNase or proteinase. This protocol has been applied successfully for labeling zebrafish embryos up to 24 hrs old with the *pax [ZF-a]*, *pax [ZF-b]* (Krauss et al., *Nature* **353**:267-270, 1991; Püschel et al., *Development* **114**:643-651, 1992), zebrafish *Krox-20* (provided by Oxtoby and Jowett), *Zfcad-1* (Joly et al., *Differentiation* **50**:75-87, 1992) and *Axial* (Strähle et al., in preparation) probes. We employed this protocol to doubly label zebrafish embryos with two RNA probes simultanously. In addition, due to its gentle treatment, many antigenic determinants survive, enabling subsequent detection of antigenic markers by immunohistochemistry (tested with the anti-Brachyury (Schulte-Merker et al., *Development*, in press; 1992), anti engrailed/invected 4D9 (Patel et al., *Cell* **58**:955-968), zn-12 and zn-8 (Trevarrow et al., *Neuron* **4**:669-679, 1990) antibodies.

Fixation

BT-Fix (Store in fridge up to a week)

4% paraformaldehyde
4% sucrose
0.12 mM $CaCl_2$
0.1 M NaPi pH 7.4

• Fix embryos still in chorions in BT-Fix over night at 4°C. Remove chorions next day with sharp watchmaker forceps.

• For long term storage dehydrate embryos through an ethanol series (30% in PBS, 50% in PBS, 70% in H_2O 3-5 min each step). Store in freezer (-20°C). Embryos remain ok up to at least several months.

Preparation of digoxygenin-labelled antisense RNA probes

RNA probes can be made using the components of the Boehringer Mannheim kit but for those who believe in making their own buffers the details of the procedure are given below.

Reaction mix:

linearized template DNA (0.5 mg/ml)	2.0 ml
NTP labeling mix	2.0 ml
10X transcription buffer	2.0 ml
RNase inhibitor (20 units/ml)	0.5 ml
dH_2O	12.5 ml
RNA polymerase (20 units/ml)	2.0 ml

• Incubate at 37°C for 2 hrs.

• Add 2 ml DNase 1 (20 units/ml) and incubate for 15 min.

• Add 1 ml 50 mg/ml tRNA and precipitate with 2.5 ml 4 M LiCl, 75 ml ethanol at -20°C.

• Spin 13 krpm for 5 min; wash pellet with 80% ethanol, dry, and resuspend in 50 ml TE.

• This reaction should yield about 10 mg of RNA; scaling up the reaction volume 5 times, but with the same amount of template will increase the yield of RNA to over 40 mg. We usually test our probes over a concentration range using a 1/100 to 1/300 dilution of a 10 mg scale reaction.

NTP-labelling Mix:

3.5 mM DIG-UTP
10.0 mM ATP
10.0 mM CTP
10.0 mM GTP
6.5 mM UTP
10.0 mM Tris/HCL pH 8.0

10X Transcription Buffer:

400 mM Tris 8.0
60 mM $MgCl_2$
100 mM DTT
100 mM NaCl
20 mM Spermidine

Hybridization

• Rehydrate stored embryos (70%, 50%, 30% ethanol, 3-5 min each step) or use fixed embryos immediately after removal of the chorion.

• Wash 4 times in PBT 5-10 min each.

PBT:
1x PBS
0.2% BSA
0.1% Tween 20

• Wash 10 seconds in H_2O.

• Remove H_2O well and add cold (-20°C) acetone, leave at -20°C for 7 min.

• Wash twice in PBT, 5-10 min each step.

• Transfer embryos to Eppendorf tubes.

• Remove PBT and carefully add 400 ml pre-hyb-buffer; when embryos have sunk to bottom, replace with a fresh aliquot of pre-hyb-buffer.

Pre-hyb-buffer:
50% Formamide
5x SSC
50 mg/ml Heparin
5 mM EDTA
1 mg/ml ribosomal RNA
(Sigma R 7125)
0.1% Tween20

• Incubate tubes in horizontal position with embryos. nicely spread out in the tube for 3 hrs to over night at 55°C.

• Replace with hyb-buffer (pre-hyb-buffer with DIG labeled antisense RNA, see above). Hyb-Buffer is heated to 80°C for 2 min before use. Hybridize over night as described in the pre-hybridization step.

• Wash in pre-hyb buffer 2 min at room temperature.

• Replace with fresh pre-hyb-buffer, incubate for 60 min at 55°C.

• Replace with prewarmed 50/50 pre-hyb-buffer/PBT, incubate for 30 min at 55°C, repeat this step once.

• Wash and block in PBT three times at room temperature for approx 5, 15, and 30 min.

Antibody staining

• Preabsorb alkaline phosphatase conjugated DIG antibody (Boehringer) by incubating a 1/400 dilution of the antibody with acetone powder prepared from adult zebrafish (see below) in PBT on a roller at 4°C over night.

• Dilute further to give a 1/2000 final dilution of the DIG antibody which is then applied to the blocked embryos and incubated over night at 4°C.

• Wash embryos 6 times (approx. 2, 5, 10, 20, 30, 60 min) at room temperature in PBT.

• Up to now all washes etc have been carried out in Eppendorf tubes (0.5 ml or 1.5 ml depending on the number of embryos). For staining we transfer the embryos into glass embryo dishes or 24 well tissue culture plates.

• Wash twice in AP-staining buffer.

AP-staining buffer:
(Prepare just before use.)
100 mM NaCL
50 mM MgCl
100 mM Tris pH 9.5
0.1% Tween 20
5 mM Levamisol

• Start staining reaction by adding 33 ml 25 mg/ml nitroblue tetrazolium salt (NBT) and 3.3 ml 50 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) to 1 ml AP-staining buffer containing the embryos. Incubate at room temperature in the dark. Follow staining reaction under a dissecting microscope with illumination from above against a white background.

• When satisfactory signal levels have developed, stop reaction with two washes in PBT.

• For storage, dehydrate through ethanol series (30% in PBS, 50% in PBS, 70%) and keep at -20°C. The stain does not diffuse significantly even after several months of storage.

Clearing of embryos

• Dehydrate further (85%, 95%, 100% twice) 10 min each step.

• Twice methylsalicylate 10 min each, then examine under dissecting scope or mount in permount.

• The blue AP reaction product is not very stable in methylsalicylate. For storage, embryos can be transferred back into ethanol.

As an alternative to the above clearing protocol, especially if embryos are not going to be mounted permanently, use the following protocol.

• Transfer embryos into methanol (10 min).

• Soak 10 min isopropanol.

• Rinse twice for 10 min each in 1.2.3.4.tetrahydronaphtalene, then analyze using the dissecting scope.

• For analysis with the compound microscope, mount embryos on coverslips in 1/1 canada balsam/ methylsalycilate, this however results in loss of transparency of the yolk.

• The embryos can be stored by transfer into mineral oil (Sigma) for a long time and can simply be put back into tetrahydronaphtalene for examination.

Preparation of acetone powder for preabsorbing the DIG antibody

• Sacrifice adult zebrafish by treatment in 40 mg/ml MESAB, pH 7.

• Freeze fish in liquid nitrogen and grind them under nitrogen in mortar to fine powder.

• Transfer powder into centrifuge tube, add cold acetone and keep on ice for 30 min. Shake vigorously every now and then.

• Spin in cooled Sorvall centrifuge (HB4 rotor), 10 krpm for 10 min.

• Resuspend pellet in ice cold acetone, keep on ice for 10 min, shake occassionally.

• Repeat centrifugation and resuspend in acetone.

• Acetone powder can be stored at -20° C in acetone or it can be dried by spreading on a filter paper and kept as dried powder in the fridge in a sealed vial.

• For preabsorption resuspend an aliquot of the dried powder in PBT and add DIG antibody. Incubate at 4° C over night.

Sectioning of stained embryos

To improve the resolution of the analysis, it is sometimes desirable to cut sections from DIG-labeled embryos. We found it most convenient to embed embryos in wax:

• Melt fibrowax at 60°C over night.

• Dehydrate embryos in ethanol (30%, 50%, 70%, 80%)

• Treat with 80% ethanol/n-butanol (3/1), 90% ethanol/butanol (1/1), 100% ethanol/n-butanol (1/3), n-butanol, fibrowax/butanol (1/1) at 60°C and twice in wax at 60°C each step for about 15 minutes with the exception of the n-butanol treatment (20 min).

• Transfer embryos into mold and fill mold with wax. Embryos can be oriented with some practice in the mold. Alternatively, putting many embryos in one block will usually result in at least one in the desired orientation. Make sure that the wax is not heated over 60°C as this makes it brittle after solidifying, resulting in poor sections.

• After solidifying, wax blocks are mounted on holders and excess wax is trimmed off.

• Cut ribbons of 8 m sections on a standard microtome.

• Transfer ribbons to preheated Tespa-coated slides with a drop of water on a heating plate set to about 55°C. Set the ribbon afloat on the water. Careful heating and monitoring is required at this step because the wax needs to be heated sufficiently to stretch out, but it should not melt completely. When the sections have spread out nicely, put cardboard between the heating plate and the slide carrying the sections to reduce the heat. Allow the water to dry off evenly over night.

• When slides are completely dry, dewax them by submersing them twice in Histoclear (National Diagnostics). Then add a few drops of Permount (Fischer) and coverslip.

CELL CYCLES, CLONAL STRINGS, AND THE ORIGIN OF THE ZEBRAFISH CENTRAL NERVOUS SYSTEM

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We examined developing lineages of central nervous system (CNS) progenitor cells during gastrulation and early segmentation in the zebrafish embryo, and observed consistent coupling of specific morphogenetic behaviors with particular cell cycles. The cells divide very synchronously. Their divisions become progressively oriented, and act synergistically with oriented intercalations during the interphases of zygotic cell cycles 15 and 16 to extend a single lineage into a long, discontinuous string of cells aligned with the nascent embryonic axis. Convergence brings the string to the dorsal midline, and once there, the cells enter division 16. This division and following intercalations convert the single string into a bilateral pair of strings, one forming a part of each side of the neural tube. The stereotyped cellular behaviors appear to account for the previously reported clonal restriction in cell fate, and to underlie morphogenesis of a midline organ of proper length and bilateral shape. Regulation of cellular morphogenesis could be cell-cycle dependent. (In preparation)

EXPANDED MIDLINE SIGNALING IN THE ZEBRAFISH ANTERIOR CENTRAL NERVOUS SYSTEM

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In all vertebrates the brain develops from the enlarged anterior part of the neural plate. However, in the zebrafish mutant *cyclops* the girth of the central nervous system (CNS) is nearly uniform along its length. Changes in expression patterns of homeobox genes and neuronal markers reveal that the entire ventral forebrain is missing, as well as its precursor region in the neural plate. The massive deletion is due to a nonautonomous action of the mutation: as few as one wild-type cell transplanted to the midline of a mutant embryo, can rescue the forebrain phenotype including cyclopia. Establishment of forebrain ventral positional values and size may thus require inductive signaling by CNS midline cells of a “prefloor plate”, whose specification depends upon the *cyclops* gene product.

NEW PROTOCOLS FOR RAISING BABIES

By Michael B. Rust; School of Fisheries, WH 10, University of Washington, Seattle, WA 98195

Spawning Zebrafish in a Net Bottom Bucket

Zebrafish can be spawned in containers that have had their bottoms cut out and replaced with 1/4" or 1/8" black plastic netting (Vexar works well). We use 2.5 gallon plastic buckets suspended in clean temperature-controlled 10 gallon aquaria.

The eggs drop through the net to the bottom of the aquarium. Because there are no marbles, the eggs are easy to remove. The adults stay isolated from the eggs so they are also easy to remove. We have used a second bucket (with a solid bottom) around the net bottom bucket to harvest eggs.

The eggs are then incubated in a plastic 750 ml bucket which has had the bottom removed and replaced with a 250 µm nitex screen. Incubation containers are suspended in an aquarium with a heater and an active biofilter. The same container is used through the larval stage until fish are large enough to transfer out of the aquarium and be weaned onto flake food.

Zebrafish Larvae Don't Need Live Food: Rearing Larvae on Commercial Diets

If you hate culturing paramecia for the first feeding of zebrafish larvae, why not use an artificial diet? We have had excellent results rearing zebrafish larvae entirely on a yeast-based diet made by Provesta Corp. of Bartlesville, OK. Their diet, called "Microfeast", is intended to be fed to *Artemia* and rotifers, but it works well on zebrafish larvae, too.

Artificial diets are messier than live diets, so we culture the zebrafish larvae in plastic 750 ml buckets which have had the bottoms removed and replaced with a 250 µm nitex screen. A hot glue gun does a good job of attaching the screen to the bottom of the bucket. These buckets are then suspended in a heated 20 gallon aquarium with a well-aged biological filter. The diets are fed by an automatic feeder ("The Fish Sitter", available at large aquarium stores) that has been modified to feed continually through the daylight hours.

It is important not to overfeed larvae. The feeder will hold a lot more feed than is necessary. About 0.1 g/day is enough to feed 200-300 larvae in a 750 ml bucket. Daily maintenance takes about 15 minutes and consists of refilling the feeder and flushing the bucket with clean water to remove uneaten food and feces.

Marvelous Meaty Artemia *Cookies*

Instead of hatching fresh Artemia for your broodstock and juvenile zebrafish every day, your might consider making *Artemia* cookies and feeding these instead. First, hatch a big batch (about two weeks' worth) of *Artemia*.

After hatching, clean the shrimp in the normal way, rinse with fresh water, and then concentrate on a 100 µm screen. (We use a 3" PVC connector with a Nitex screen sandwiched between the fitting and a short length of 3" pipe.)

Pipette the normal ration of Artemia for a tank into the well of an ice cube tray (plastic ones that make small cubes are best). Repeat until you run out of *Artemia* or tray space. Add fresh fish water to fill the trays and freeze.

When it is time to feed the fish, just pop out a cookie (ice cube) and add to the tank. The ice will slowly melt and release the *Artemia*, feeding your fish. You can do the same thing with any fresh material such as worms, fruit flies, or liver paste.

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